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A Novel Gemini-like Cationic Lipid for the Efficient Delivery of siRNA

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Although RNAi is a promising strategy for the intervention of human diseases, an essential prerequisite is the development of low-toxic and efficient siRNA delivery systems. In this report, we describe a novel gemini-like cationic lipid (CLD) that was developed and evaluated for the delivery of siRNA. The novel lipid contains a disulfide bond in the spacer, which can be cleaved under reducing conditions in cytoplasm and decrease lipid toxicity. The liposomes formed by CLD exhibited high siRNA binding property, which was facilitated by the increasing number of cationic charges. Moreover, the cationic lipids showed low cytotoxicity in cell-based assay. They protected siRNAs from degradation by nucleases and the complexed siRNA was released efficiently in the presence of low concentrations of polyanionic heparin. High Content Screening and confocal microscopy studies demonstrated that lipoplexes with FAM labeled siRNA showed efficient cell uptake by HeLa and A375 cells. Finally, siRNA targeting MEK1 mRNA formulated in liposomes demonstrated effective endosomal escape after cellular uptake and efficient gene silencing of MEK1 expression. These results demonstrated the promising use of the novel gemini-like cationic lipid for the efficient and safe delivery of siRNA.

Introduction

RNA interference (RNAi) is a conserved cellular gene silencing mechanism by which long double-stranded RNA (dsRNA) is first cleaved to short interfering RNA (siRNAs) and then directs the degradation of complementary mRNA sequence to suppress the expression of a target gene.[1] Synthetic siRNAs with 21-23 base pairs in length, which can be prepared in large scale, have attracted great interest as a tool for the discovery and validation of gene function, and offers great potential to silence target genes in the intervention of human diseases,[2] including cancer,[3,4] infection and inflammation,[5] respiratory and neurological disorders,[6,7] and immunodeficiency.[8] Despite their high efficiency in vitro, only limited gene silencing by siRNAs have been achieved in vivo due to their rapid enzymatic degradation, poor cellular uptake and inefficient intracellular release. Development of an efficient and safe delivery system is still a key challenge for siRNA-based drug discovery.[9,10] Viral vectors are efficient delivery systems and have resulted in the first successful gene therapy,[11] but they suffer from numerous disadvantages. Compared with the viral delivery systems, non-viral delivery systems are preferred approaches because of their simple usage, limited immune response, high biocompatibility, low cost and convenient structural modification. In addition, nonviral systems can be used for the delivery of siRNAs, plasmid DNA and antisense oligonucleotides.[12] Various non-viral delivery systems have been investigated for siRNA delivery, such as cationic lipid nanoparticles,[13] chitosan nanoparticles,[14] polyaminoamine (**PAMAM**) dendrimers,[15] poly(beta-amino ester)s[16] and protein transduction domains (**PTD**s) peptides.[17] An important component of the transfection vehicle is cationic lipids, as this lipid is proposed to be responsible for both cell association (internalization) and endosomal escape. In the panorama of cationic amphiphiles, gemini surfactants are a relatively new class of molecules with unique physicochemical properties. Cationic geminilipids, consisting of 2 cationic head groups and 2 hydrophobic tails linked by an alkyl spacer, have lower critical aggregation concentrations (in the 10^{-6} rather than the 10^{-3} M range) than conventional cationic lipids with single-chain, single-head group.[18] While the structural diversity investigated to date is relatively broad, the chemical nature of these domains typically remains the same. Cationic head-groups typically are consisted of primary or tertiary amines together with polyamine and quarternary ammonium,[19,20] guanidine groups,[21] and amino acids,[22] which promote electrostatic interactions with siRNAs and cellular internalization. Cationic lipids containing amino acids, such as lysine (**Lys**) and arginine (**Arg**) with different hydrocarbon chain lengths, have been reported to influence transfection efficiency and cytotoxicity.[23,24] Steroid moiety[25] and two hydrophobic chains[26,27] are typically required for lipid bilayer stability. An explanation for the high efficiency of gemini surfactants in transfection is that the lipid aggregates have two different pKa values, one above, one below the physiological pH. As a result, the lipoplex undergoes pH-induced morphological changes in the acidified endosome that allow escape from the endosomal compartment.[28]

One of the critical factors to be addressed in the eventual gene therapy treatment is the cytotoxicity associated with the continued cytoplasmic residence and tissue accumulation of the delivery system. With this in mind, researchers have investigated the incorporation of stimuli-responsive bonds in the linker moiety, such as enzyme cleavage,[29] pH[30] or redox susceptible chemical groups,[31] which allow the metabolism of delivery carriers into nontoxic products.[32,33] Redox potential has been proposed as an efficient stimuli mechanism in gene delivery because of the high difference (10^2-10^3 fold) existing between the reducing intracellular space and the oxidizing extracellular milieu.[34] Biomaterials with disulfide covalent bond (-S-S-) are more stable in the oxidizing extracellular environment, but can be easily reduced in intracellular compartments by the redox agents such as

reduced glutathione (L-γ-glutamyl-L-cysteinylglycine, **GSH**) in the cytosol of host cells. Due to its reversibility, disulfide bond has been widely used in designing gene delivery systems.[35] We hypothesized that upon cell uptake, the degradable properties of the new gemini-like cationic lipid (**CLD**) structure would trigger endosomal dissociation and release of the therapeutic siRNAs from the liposomes.

In this report, a novel redox-sensitive gemini-like cationic lipid was designed to form lipoplexes with siRNA that can enter cells by endocytosis. The cationic lipids subsequently facilitate endosomal destabilization and undergo complete metabolism into amino acids and fatty acids. Moreover, the use of redox-sensitive amino acid cystine as spacer and linker for the gemini-like cationic lipid with oleyl ester has not been reported. The delivery of siRNA by **CLD** showed good siRNA transfection efficiency and low cytotoxicity compared with commercially available Lipofectamine RNAiMAX were also evaluated.

Experimental

Materials and cell lines. N,N′-dicyclohexylcarbodiimide (**DCC**), 4-dimethylaminopyridine (**DMAP**), Boc-Lys(Boc)-OSuc, cysteine, oleyl alcohol, and Boc_2O were purchased from GL Biochem Co., Ltd. (Shanghai, China). Heparin sulfate was purchased from Sigma-Aldrich Co., LLC. (Shanghai, China). The Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) was used as suggested by the manufacturer. siRNA targeting MEK1 mRNA (**siMek1**, antisense strand, 5′-AGC AUG AAC CAU GAG UUG CdTdT-3′, sense strand, 5′-GCA ACU CAU GGU UCA UGC UdTdT-3′) was supplied by Guangzhou RiboBio Co., Ltd. (China). FAM-labeled siRNA (FAM-**siMek1**, antisense strand, 5′-FAM-AGC AUG AAC CAU GAG UUG CdTdT-3′, sense strand, 5′-FAM-GCA ACU CAU GGU UCA UGC UdTdT-3′) was supplied by Shanghai GenePharma Co., Ltd. (China). Dulbecco′s modified Eagle′s medium (**DMEM**) and fetal bovine serum (**FBS**) were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade.

Human cervical carcinoma cells HeLa, human maligmant melanoma cells A375, human embryonic kidney cells HEK293 (Institute of Material medical, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) were used in the in vitro assay models. All the cells were grown continuously as a monolayer in **DMEM** in a humidified incubator at 37 ^oC and under 5% CO_2. **DMEM** was supplemented with 10% heat-inactivated FBS.

Synthesis of di((Z)-octadec-9-en-1-yl)3,3'-disulfanediylbis(2-(2,6-diaminohexanamido) propanoate) (CLD): **CLD** was prepared from cysteine **1**. Briefly, as shown in Scheme 1, the synthesis strategy was started with construction of the protected cystine **2**, followed by coupling with oleyl alcohol to give **3**, and the Boc protecting group of **3** was subsequently removed in the presence of 30% CF_3CO_2H/CH_2Cl_2 to obtain **4**. Compound **4** was conjugated with Boc-Lys(Boc)-OH to obtain **5**, and then Boc protecting group was removed in the presence of 30% CF_3CO_2H/CH_2Cl_2 to give **6** (**CLD**).

The structure of product **6** was determined by ^1H NMR and ^{13}C NMR spectroscopy (Bruker AVANCEIII 400, Billerica, MA, USA). The solvent used to dissolve **CLD** was DMSO-d_6. The accurate mass of **CLD** was also confirmed by ZABHS mass spectrometry (VG Scienta, England).

(A) Synthesis of L-Boc-cystine 2. L-cystine (1.0 g, 4.16 mmol) and NaOH (166 mg, 4.16 mmol) were dissolved in 5 mL distilled water. After reacting for 10 min, a clear solution was formed, and the mixture was cooled to 0 ^oC. A solution of Boc_2O (2.0 g, 9.15 mmol) in 5 mL 1,4-dioxane was added dropwise in about 1 h and the mixture was stirred at room temperature overnight. The mixture was then acidified to pH 2 with 1 N HCl/H_2O, 20 mL of brine was added, and the solution was extracted with ethyl acetate three times (20 mL each). The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to yield 1.56 g (yields: 85.0%) of Boc-cystine as a white solid. ^1H NMR (400 MHz, CD_3OD) δ 4.45 (q, 2H, -CH_2**CH**-), 3.30-2.95 (m, 4H, -S**C**H_2-), 1.47 (s, 18H, -**C**H_3 in *t*-B_{OC} group).

(B) Synthesis of 3. Boc-cystine (1.0 g, 2.27 mmol), **DCC** (1.124 g, 5.45 mmol) and 4-dimethylamiopryidine (0.25 g, 2.27 mmol) were dissolved in anhydrous CH_2Cl_2. After 5 min, oleyl alcohol (1.46 g, 5.45 mmol) was added to the solution and stirred at room temperature overnight. The reaction mixture was then filtered through 1cm Celite, the filtrate was collected and separated by a silica column using cyclohexane/ethyl acetate 20:1 (v/v). This procedure produced 1.39 g (yields: 65.0%) of **3** as colorless oily liquid. ^1H NMR (400 MHz, $CDCl_3$) δ 5.46-5.28 (m, 4H, =**CH**-), 4.57 (d, 2H, -**CH**CO_2-), 4.18-4.20 (t, 4H, -O**C**H_2-), 3.17 (d, 4H, -S**C**H_2-), 2.11-1.91 (m, 8H, -**C**H_2CH=), 1.71-1.59 (m, 4H, -OCH_2**C**H_2-), 1.44 (s, 18H, -**C**H_3 in *t*-B_{OC} group), 1.40-1.04 (m, 44H, -**C**H_2- in long chain), 0.87 (t, 6H, -**C**H_3 in long chain). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7 (-**C**O_2-), 155.0 [-(**C**=O)NH-], 129.9 (-**C**H=), 129.7 (=**C**H-), 80.1 (-**C**O- in *t*-B_{OC} group), 65.9 (-O**C**H_2-), 53.1 (-**C**HCO_2-), 41.5 (-S**C**H_2-), 32.5, 31.9, 29.8, 29.7, 29.6, 29.5, 29.3, 29.2, 28.5, 28.3, 27.2, 25.8, 22.6 (-**C**H_2- in long chain), 14.1 (-**C**H_3 in long chain). MS: Calcd. for $C_{52}H_{96}N_2O_8S_2$ $[M+H]^+$ m/z 941.7; found 941.7.

(C) Synthesis of 4. Compound **3** (1.20 g, 1.27 mmol) was dissolved in 6 mL CH_2Cl_2. After a clear solution was formed, the solution was cooled in ice bath while 6 mL of 30% CF_3CO_2H/CH_2Cl_2 was added. The solution was stirred until the reaction mixture reached room temperature (in about 30 min), then the solvent was removed slowly under reduced pressure, and the residue was treated with ethyl acetate, and dried under vacuum overnight to give **4** (0.90 g, 69.3%) as yellow oil. ^1H NMR (400 MHz, $CDCl_3$) 5.37-5.24 (m, 4H, =**CH**-), 4.10 (t, 4H, -O**C**H_2-), 3.88-3.73 (m, 2H, -**C**HCO_2-), 3.48-2.83(m, 4H, -S**C**H_2-), 2.05-1.94 (m, 8H, -**C**H_2CH=), 1.66-1.21 (m, 48H, -**C**H_2- in long chain), 0.84 (t, 6H, -**C**H_3 in long chain). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7, 170.5 (-**C**O_2-), 129.9 (-**C**H=), 129.8 (=**C**H-), 63.0(-O**C**H_2-), 53.4, 50.7 (-**C**HCO_2-), 40.3 (-S**C**H_2-), 32.8, 32.6, 31.9, 29.8, 29.7, 29.4, 29.3, 29.2, 28.5, 28.4, 28.3, 27.2, 25.7, 22.6 (-**C**H_2- in long chain), 14.1 (-**C**H_3 in long chain). MS, Calcd. for $C_{42}H_{80}N_2O_4S_2$ $[M+H]^+$ m/z 741.6; found m/z 741.6.

(D) Synthesis of 5. Compound **4** (0.50 g, 0.67 mmol), DCC (1.12 g, 5.45 mmol) and DMAP (0.25 g, 2.27 mmol) were dissolved in anhydrous CH_2Cl_2. After 5 min, Boc-Lys(Boc)-OH (0.51 g, 1.47 mmol) was added to the solution and stirred at room temperature overnight. The reaction mixture was then filtered through 1cm Celite, and the filtrate was purified on a silica column using

cyclohexane/ethyl acetate 4:1 (v/v) to afford 0.48 g (yields: 51.0%) of **5** as colorless oily liquid. ^{1}H NMR (400 MHz, CD_3OD) δ 5.44-5.32 (m, 4H, =**CH**-), 4.84-4.54 (m, 2H, -SCH_2**CH**-), 4.17 (t, 4H, -O**C**H_2-), 3.53-3.03 (m, 8H, -S**C**H_2- and -(C=O)NH**C**H_2-), 2.12-1.30 (104H, -**C**H_2- in long chain and in Lysine part, -CH_3 in *t*-B_{OC}), 0.92 (t, 6H, -**C**H_3 in long chain). ^{13}C NMR (101 MHz, CD_3OD) δ 173.7 [-(**C**=O)NH-], 170.3 (-CO_2-), 158.4, 157.1 [-(**C**=O)- in *t*-B_{OC}], 129.5 (=**C**H-), 129.4 (-**C**H=), 79.2, 78.4 [-**C**O- in *t*-B_{OC}], 65.5 (-CO_2**C**H_2-), 54.5 (-NH**C**H-), 51.7 (-**C**HCO_2-), 39.7 (-S**C**H_2-), 39.4 (-NH**C**H_2-), 33.4, 32.3, 31.7, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 28.9, 28.3, 27.5, 26.8. 25.6, 25.4, 24.7, 22.7, 22.4(-**C**H_2- in long chain and in Lysine part, -**C**H_3 in *t*-B_{OC}), 13.2 (-**C**H_3 in long chain). MS, Calcd. For $C_{74}H_{136}N_6O_{14}S_2$ $[M+H]^+$ m/z 1400; Found 1400.

(E) Synthesis of 6. Compound **5** (0.30 g, 0.25 mmol) was dissolved in 6 mL anhydrous CH_3OH. After a clear solution was formed, the solution was cooled in ice while 6 mL 30% CF_3CO_2H/CH_2Cl_2 was added. The solution was stirred until the reaction mixture reached room temperature (in about 30 min), then the solvent was removed slowly under reduced pressure. The solid was re-dissolved in CH_3OH, and addition of diethyl ether precipitated **6** (0.23 g, 92.0%) as yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.49 [s, 2H, -(C=O)**NH**-], 5.41-5.27 (m, 4H, =**CH**-), 4.61-4.25 (m, 2H, -HN**CH**CO_2-), 4.11-3.98 (m, 4H, -O**C**H_2-), 3.24-3.01 (m, 6H, -S**C**H_2- and H_2N**C**H-), 2.78-2.66 (m, 4H, H_2N**C**H_2-), 2.05-1.15 (m, 68H, -**C**H_2- in long chain and in Lysine part), 0.85 (t, 6H, -**C**H_3 in long chain). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.3 [-(**C**=O)NH-], 169.4 (-CO_2-), 130.1 (-**C**H=), 130.0 (=**C**H-), 65.6 (-O**C**H_2), 52.1, 52.0 (-**C**HCO_2-), 38.7, 38.6 (-S**C**H-), 31.7 (-COCH**C**H_2-), 30.6, 29.5, 29.4, 29.3, 29.2, 29.1, 28.5, 28.4, 27.1, 27.0, 26.6, 25.7, 22.6, 21.3, 21.2, 21.1 (-**C**H_2- in long chain and Lysine part), 14.4 (-**C**H_3 in long chain). HRMS: Calcd. for $C_{54}H_{104}N_6O_6S_2[M+H]^+$: m/z 997.7531; found 997.7506.

Preparation of CLD liposomes and siRNA loading. The cationic **CLD** liposomes were prepared by a thin-film hydration method. **CLD** (4.2 mg) were dissolved in ethanol/chloroform (1:1). After removal of solvent by a stream of nitrogen, the residual solvent was removed by drying overnight, to form a thin lipid film. Subsequently, 1.0 mL sterile milliQ water was added to the dried lipid film and sonicated at room temperature, then extruded through 0.22 μm pore diameter polycarbonate membrane filters using a syringe. The **CLD** liposomes were stored at 4 ^{o}C until use.

For transfection experiments, cationic **CLD** liposomes and **siMek1** were mixed at different charge ratios (N/P ratio). **CLD** liposomes were diluted to different concentrations to obtain the desired N/P ratio using Opti-MEM medium (Invitrogen, CarIsbad, USA) and **siMek1** solution (50 nM in RNAase free water) was subsequently added to the formulation and mixed by pipetting. The **CLD** lipoplexes were incubated at room temperature for 30 min before use.

Characterization of CLD liposome. Ethanol/chloroform (v/v = 1:1) solution of **CLD** (4.2 mg/mL) was dried in glass test tubes by rotary evaporation under reduced pressure followed by high vacuum overnight. The lipid film was rehydrated with 1 mL deionized water and sonicated for 30 min to form liposomes. The obtained **CLD** liposomes were subjected to extrusion one time through a polycarbonate membrane filter of 0.22 μm pore size (Whatman, Gentaur Molecular Products, Belgium). Particle size (diameter, nm) and surface charge (zeta potential, mV) of the **CLD** liposomes were obtained from three repeat measurements by Zeta sizer Nano S (Malvern instruments, UK).

For scanning electron microscopy (SEM) analysis, the liposome solution was dropped on the silicon slice, vacuum dried for one day, and stained with gold powder. The SEM images were recorded by Hitachi S-4800 (Hitachi, Ltd., Japan) with 6 KV accelerating voltage.

Gel retardation assay. CLD lipoplexes with different N/P ratios, ranging from 1 to 12 (**siMek1** concentration was fixed at 20 μM), were prepared as above. The formation of **CLD**/**siMek1** lipoplexes was confirmed by gel electrophoresis on a 1% agarose gel. After incubation at room temperature for 30 min, **CLD**/**siMek1** lipoplexes with different N/P ratios were loaded with 6 × loading buffer (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) onto the agarose gel, and electrophoresis was carried out at 110 mV for 30 min in 1 × tri-acetate-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA) (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China), and visualized using a UV illuminator (ChemiDoc XRS System, BIO-RAD, USA) with Goldenview^{TM} (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) staining. Free **siMek1** was used as a control.

Serum stability assay. CLD lipoplexes with different N/P ratios, ranging from 4 to 12 (**siMek1** concentration was fixed at 20 μM), were prepared as above. The formation of **CLD**/**siMek1** lipoplexes was confirmed by gel electrophoresis on a 1% agarose gel. Samples were mixed with FBS in 1:1 volume ratio to give 50% serum concentration and incubated at 37 ^{o}C for 24 h, **CLD**/**siMek1** lipoplexes with different N/P ratios were loaded with 6 × loading buffer (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) onto the agarose gel, and electrophoresis was carried out at 110 mV for 30 min in 1 × tri-acetate-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA) (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China), and visualized using a UV illuminator (ChemiDoc XRS System, BIO-RAD, USA) with Goldenview^{TM} (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) staining. Free **siMek1** was used as a control.

Model siMek1 displacement assay. The ability of **CLD** lipoplexes to release **siMek1** after a challenge with the competing polyanionic heparin was determined using complex stability assay. Lipoplexes with **CLD**/**siMek1** mass ratio of 11:1 (N/P = 12) were prepared to ensure complete binding of **siMek1** by **CLD** liposomes, and then incubated with 1.25, 6.25, 12.5, 18.8, 25.0, 31.3 μg/μL of heparin sulfate in RNase free water at room temperature for 15 min. The samples were analyzed on a 1% agarose gel as described above. Results were presented as the average of three independent experiments at least.

Cell viability assay. A CCK-8 assay (cell counting kit-8,

Dojindo Laboratories, Kumamoto, Japan) was used to assesss the cytotoxicity of **CLD** liposomes against the HeLa and HEK293 cells according to the manufacturer's protocol. Briefly, the cells were seeded on a 96-well plate at a density of 1×10^4 cells/well and incubated in **DMEM** at 37 ^oC under 5% CO_2/95% air atmosphere overnight. Cells were then incubated with the **CLD** liposomes at different N/P ratios (6, 8, 10, 12, 14, 16, 18, 20, 22, and 24) for another 24 h. The CCK-8 assay solution of 10 μL (10%, v/v) was added to each well of the plate. After incubation for 2.5 h at 37 ^oC under 5% CO_2/95% air atmosphere, the UV absorbance was measured at 450 nm/630 nm using Flex Station 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices, CA, USA). Mean cell viability was calculated using the formulation: [(RA - RE)/(RB - RE)] × 100% (RA, RB, RE were defined as the absorbance of experimental samples, untreated samples and blank controls, respectively).

In vitro cellular uptake and intracellular translocation of siRNA. To measure the ability of **CLD** lipoplexes to transfer **siMek1** into HeLa cells and A375 cells, a concentration of 50 nM FAM-labled **siMek1** was formulated in the **CLD** liposomes at different N/P ratios (6, 8, 10, 12, 14, 16, 18, 20, 22, and 24) according to the above method. HeLa/A375 cells were seeded in 96-well plates at a density of 6×10^3 cells/well and incubated in DMEM at 37 ^oC under 5% CO_2/95% air atmosphere overnight. Cells were then transfected with either **CLD** lipoplexes or Lipofectamine RNAiMAX. Following the treatment, cells were washed with PBS three times, and then fixed with 4% paraformaldehyde (PFA, Invitrogen, CA, USA) in PBS for 15 min. Cells were washed again with PBS three times, and then 4′,6-diamidino-2-phenylindole dithdrochloride in PBS was added to each well (**DAPI**, Thermo Scientific, Rockford, USA) and incubated for 15 min to stain the cell nucleus. After washing away the excess **DAPI**, cellular uptake of FAM-labeled **siMek1** was detected by Cellomics ArrayScan® VTI High content screening Reader (Thermo Fisher, Rockford, USA) at standard acquisition mode with a 10X objective. The exposure time for channel 1 (**DAPI**) and channel 2 (**5-FAM**) were set when photo-sensibility of the brightest pictures was no more than 30%. The data were analyzed with the Target Activation BioApplication of Thermo Scientific Cellomics High Content Informatics (HCi^{TM}, Thermo Fisher, Rockford, USA). The background correction parameters were set at > 105 pixels, larger than a cell's dimension. Confocal fluorescent microscopy was used to assess the intracellular trafficking of **siMek1**. FAM-labeled **siMek1** was mixed with **CLD** liposomes at the N/P ratio of 12. Following incubation of HeLa cells (1×10^5 cells per dish) in glass-bottomed dishes in **DMEM** at 37 ^oC under 5% CO_2/95% air for 24 h, media containing lipoplexes were added (the final concentration of **siMek1** was 50 nM) to each dish and transfected for another 5 h. Following the treatment, cells were washed with PBS buffer two times, and 4% **PFA** in PBS buffer was the added to the dish and incubated for 15 min. After washing away the excess **PFA**, cells were mounted with anti-photobleaching mounting media with **DAPI** for 15 min. The fluorescent images of the cells were analyzed using a TCS SP2 confocal microscope (Leia, Germany). The 492 nm line of a 30 mW tunable argon laser was used for the excitation of FAM, and a 25 mW diode UV 345 nm laser for **DAPI**. Emission was filtered at 518 nm and 455 nm for FAM and **DAPI**, respectively.

In vitro target gene silecning assay. For cellular uptake studies, HeLa cells were seeded in 6-well plates at a density of 2×10^5 cells/well and incubated in **DMEM** at 37 ^oC under 5% CO_2/95% air overnight. The following day, cells were transfected with **CLD** lipoplexes (with different N/P ratios of 20, 24, 28) or Lipofectamine RNAiMAX lipoplexes at **siMek1** concentration of 50 nM for 0.5, 1, 2, 4 h. Cells were washed with PBS buffer followed by incubation with **DMEM** at 37 ^oC under 5% CO_2/95% air for another 47.5, 47, 46, 44 h, respectively. After a total of 48 h transfection, the mRNA expression level of MEK1 protein was evaluated by reverse transcription polymerase chain reaction (RT-PCR). Real-time polymerase chain reaction (real-time PCR) was performed using an Mx3005P QPCR Systems (Agilent, USA). Total RNA was extracted from the cells with Trizol reagent (Invitrogen, Carlsbad, CA) from cell lysis via a standard chloroform-extraction protocol. The first-strand complementary DNA (cDNA) was reversely transcribed from RNA using the ReverTra Ace® qPCR RT Kit (TOYOBO, Japan). In brief, an aliquot of 1 μg of total RAN was reversely transcribed by ReverTra Ace transcriptase (TOYOBO, Japan) using the random primers according to the manufacturer's instructions. Real-time polymerase chain reaction (real-time PCR) was performed using an Mx3005P QPCR Systems (Agilent, USA). The reaction system was 10 μL 2 × Brilliant II SYBR Green QPCR Master Mix (600828, Agilent Technologies, USA), 0.5 μL of 20 μM of each primer, 0.05 μg of Cdna (1μL) and 8 μL of nuclease-free water. For MEK1, the sense primer sequence was 5′-CCT TGA GGC CTT TCT TAC CC-3′, and the anti-sense primer sequence was 5′-CCC ACG ATG TAC GGA GAG TT-3′. For β-actin, the sense primer sequence was 5′-CCA ACC GCG AGA AGA TGA-3′, and the anti-sense primer sequence was 5′-CCA GAG GCG TAC AGG GAT AG-3′ (RiboBio Co., Ltd, Guangzhou, China). Reaction parameters were as follows: at 95 °C for 5 min, then 40 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. RT-PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. β-actin was used as a control.

Western blot ananlysis. Cells were collected at 48 h after siMek1 transfection (30 nM). Cells were lysed in 1mL lysis buffer (Beyotime Institute of Biotechnology, Suzhou, China) for 1 h on ice and vortexed every 15 min. The lysates were clarified by centrifugation for 10 min at 13000 rpm. Protein concentration was determined using Flex Station 3 Benchtop Multi-Mode Microplate Reader at 562 nm (Molecular Devices, CA, USA). Total protein was separated on 10% Bis-Tris-polyacrylamide gels and then transferred to PVDF membranes (Millipore, Bedford, MA, USA) at 400 mA for 1 h. After blocking with 5% skim milk in Washing buffer for 1 h, the membrane was incubated with a mouse anti-MEK1 monoclonal antibody (1:1000) (Cell Signaling Technology, MA, USA) in primary antibody dilution buffer at 4 ^oC overnight, washed and incubated with a goat-anti-mouse monoclonal antibody (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% skim milk for 3 h. Finally, protein products were analyzed by Image Lab^{TM} software (ChemiDoc^{TM}

XRS System, BIO-RAD, CA, USA). Expression levels of MEK1 protein were normalized against GAPDH protein expression levels.

Statistical analysis: Analysis was performed using the Statistical Program for Social Science (SPSS) for windows (SPSS Inc., IL, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Preparation of CLD. The synthesis procedure of **CLD** monomer compound **6** was based on the standard liquid phase Boc synthesis strategy (Scheme 1) in five steps. First, the protected cystine **2** was prepared and it was conjugated with oleyl alcohol using **DCC** and **DMAP** as coupling reagents to obtain **3** (65.0%). Compound **3** was then deprotected by 30% CF_3CO_2H/CH_2Cl_2, producing **4** (94.0%). Compound **4** was conjugated with Boc-Lys(Boc)-OH using **DCC** and **DMAP** as coupling reagents, affording the intermediate compound **5** (51.0%). The N-Boc group of **5** was then deprotected by 30% CF_3CO_2H/CH_2Cl_2 to give **6** (92.0%). The ^{1}H NMR and ^{13}C NMR spectra of **6** were shown in Supplementary Fig. S1A and S1B. The characteristic peaks of **6** were all identified in the spectra. In addition, the mass spectrum of compound **6** (Supplementary Fig. S1C) exhibited a single and sharp peak at m/z 997.75062, which corresponds to the $[M + H]^+$. All these results confirmed the structure of **CLD** monomer **6**.

Characterization of CLD liposomes. The particle size of **CLD** liposomes was approximately 119.13 ± 3.56 nm and the polydispersity was 0.26 ± 0.046 (Figure 2A). The zeta potential result suggested that the net surface charge on the **CLD** liposomes was positive 57.13 ± 0.69 mv, which was expected because of the cationic amine groups in **CLD**. The size, along with positive charge from surface exposure, confirmed that we have prepared the nanoparticles of **CLD** liposomes as designed. The shape of **CLD** liposomes was characterized by scanning electron microscopy (SEM) images, demonstrating that liposomes adopt a spherical shape with an average diameter of 100 nm (Fig. 2B and 2C).

siMek1 binding property of the CLD liposomes and serum stability of CLD/siMek1 lipoplexes. Agarose gel electrophoresis was utilized to determine **siMek1** binding ability of the **CLD** liposomes, based on the disappearance of free siRNA bands on the agarose gel. **CLD**/**siMek1** lipoplexes were prepared at different N/P ratios (1, 2, 4, 6, 8, 10 and 12). As shown in Fig. 3, the **CLD** liposomes were capable of forming complexes with siRNA in a charge-dependent manner (increasing N/P ratio of siMek1 and **CLD** liposomes), resulting in retardation or disappearance of **siMek1** bands on the agarose gel. We observed decreased free **siMek1** bands with increased N/P ratios. When the N/P ratio reached 6, the migration of **siMek1** was clearly retarded. When the N/P ratio was 12, the migration of **siMek1** was completely retarded by **CLD** liposomes. From these results, we concluded that at the charge ratios of ≥ 12 **CLD** liposomes formed stable complexes with **siMek1** molecules.

In addition, we observed **CLD/siMek1** lipoplexes could effectively protect **siMek1** from degradation in 50% serum solution with increased N/P ratios (8, 10 and 12). As shown in Supplementary Fig. S2, there were brightness bands at N/P ratios of 8, 10 and 12 after 24 h. In contrast, there were no siRNA bands at N/P ratios of 4 and 6. Overall, the results of serum stability indicated that **CLD** could improve stability of siRNA in serum.**Stabilities of CLD/ siMek1 lipoplexes by heparin displacement assay.** We investigated the stability of **CLD**/ **siMek1** lipoplexes in vitro by a heparin displacement assay. Polyanions of the cell surface proteoglycans, such as heparin sulfate, have the potential to disrupt the electrostatic interactions between cationic agents and siRNA effectively.[36] **siMek1** was released from the **CLD** lipoplexes in a concentration-dependent manner as shown in Fig. 4. With increasing amounts of heparin, the intensity of upper band (**CLD**/**siMek1** lipoplexes) was decreased, and the intensity of lower band (free **siMek1**) was increased. At 31.25 μg/μL of heparin, **CLD**/**siMek1** lipoplexes appeared to be completely dissociated. The result suggests that the specific interactions between **siMek1** and **CLD** liposomes could be dissociated upon interactions with cell surface polyanions in vivo. The released **siMek1** from lipoplexes could participate in the subsequent RNAi mechanism to inhibit target gene expression.

Cytotoxicity of CLD liposomes. One of the most serious problems of cationic transfection agents is their toxicity caused by the formation of nanoscale holes in cell membrane. In this study, we investigated the cytotoxic concentration range of **CLD** liposomes. The viability of Hela and HEK293 cells was evaluated in the presence of liposomes (Fig. 5A and 5B). Although the amounts of liposomes was increased approximately six-fold, no significant decrease in cellular viability was observed comparing with untreated cells, which indicated **CLD** liposomes were not toxic in the tested concentration range. These data provided preliminary evidence for the low toxicity of the novel **CLD** liposomes for siRNA delivery.

In vitro siMek1 uptake study. High-content screening (HCS) employs fluorescent indicators to determine cellular **siMek1** delivery efficiency. For the **CLD** lipoplexes, the FAM-**siMek1** cellular uptake study was performed in Hela and A375 cells. **CLD**/FAM-**siMek1** lipoplexes were formulated at various N/P ratios. Lipofectamine RNAiMAX (LRM)/FAM-**siMek1** complex and free FAM-labeled **siMek1** were used as positive control and negative control, respectively. As demonstrated by high-content screening analysis (HCA) (Fig. 6), the delivery efficiency of the **CLD**/FAM-**siMek1** lipoplexes was dependent on the N/P ratio. The transfection efficiency increased with the increase of N/P ratio up to 24. The cells treated with free **siMek1** showed background fluorescence. Compared with the LRM/FAM-**siMek1** complexes, the fluorescence-labeled particles were uniformly distributed in cells transfected with **CLD**/FAM-**siMek1** lipoplexes. And the average fluorescence intensity of HeLa cells transfected with **CLD** lipoplexes was higher at N/P ratios ranging from 12 to 24 (Fig.7b). Moreover, there was no significant difference in fluorescence intensity in cell nucleus, which was calculated by HCA (Fig. 7a), between control groups

and groups treated with lipoplexes. This may indicates the low toxicity of our liposomes for **siMek1** delivery. Similarly, as demonstrated by HCS (Supplementary Fig. S4), the average fluorescence intensity of A375 cells treated with **CLD** lipoplexes (Supplementary Fig. S5b) was higher at N/P ratios ranging from 8 to 24, compared with the LRM/FAM-**siMek1** complexes. And there was also no significant difference in the fluorescence intensity of cell nucleus (Supplementary Fig. S5a) between control groups and lipoplexes treated groups.

The transfection efficiency at different transfection times. Transfection efficiency after transfecting for different periods of times was evaluated for the **CLD**/**siMek1** lipoplexs. The **CLD**/**siMek1** lipoplexes were formulated at different N/P ratios based on the results of **siMek1** uptake study. Lipofectamine RNAiMAX/**siMek1** and **CLD** liposomes were used as positive and negative control, respectively. We evaluated the silencing efficiency of lipoplexes at different transfection times. As demonstrated by RT-PCR in Fig. 8, efficient MEK1 mRNA knockdown by **CLD**/**siMek1** lipoplexes was observed from 0.5 h and the efficiency was improved with the increasing transfection times (from 19.7 ± 0.04% at 0.5 h to 71.5 ± 0.04% at 4 h when the N/P ratio was 28). We also found the knockdown efficiency was increased with increasing N/P ratios. The N/P ratio of 28 showed the highest knockdown efficiency. After 1 h of transfection by **CLD**/**siMek1** lipoplexes with the N/P ratios of 24 and 28, 50% of MEK1 mRNA was knockdowned, which was almost the same as the commercially availbale transfection agent Lipofectamine RNAi^{MAX} (LRM). After transfecting for 4 h, only 28% of MEK1 mRNA was left in the **CLD**/**siMek1** lipoplexes treated cells, and so was the LRM/**siMek1** complexes treated cells.

Observation of CLD/siMek1 lipoplexes in cells by confocal microscopy. To confirm the delivery of **CLD**/**siMek1** lipoplexes into cells and study their distribution inside the cells, the confocal microscopy was used to examine the cells. As shown in Fig. 9, the fluorescence signal of FAM-labeled **siMek1** was clearly detected in the cytoplasm, and no fluorescence signal was observed in cell nucleus, which indicated that **siMek1** was efficiently delivered into the cells by the **CLD**/**siMek1** lipoplexes.

Gene silencing by CLD/siMek1 lipoplexes. Gene silencing efficiencies of **CLD**/**siMek1** lipoplexes were determined after transfection of siMek1 complexes in Hela cells. **CLD** liposome alone was used as negative control. Lipofectamine RNAiMAX/**siMek1** was used as a positive control due to its well-characterized and potent transfection property. The relative MEK1 mRNA levels of treated cells with different N/P ratios (20 and 24) at the **siMek1** concentration of 50 nM are shown in Figure 11. The **CLD**/**siMek1** lipoplexes showed potent and dose-dependent (**CLD** liposomes) knockdown of MEK1 mRNA after transfection for 24h, compared with the negative controls using naked **siMek1** and **CLD** liposomes (Supplementary Fig S6). Furthermore, we measured the persistence of gene silencing induced by **CLD**/**siMek1** lipoplexes in vitro. The results (Fig. 10) showed that the MEK1 silencing efficiency of **CLD**/**siMek1** lipoplexes increased with the transfection time up to 48 h, while the commercial transfection agent (LRM) **siMek1** complexes showed decreasing silencing efficiency at 48 h transfection time. Higher N/P ratios improved the knockdown efficiency. On the protein expression level, the **CLD**/**siMek1** lipoplexs with N/P ratio of 28 showed the same MEK1 knockdown ability as LRM/ **siMek1** complexes. The western blot results (Fig. 11) indicated that, using GAPDH protein as a reference, the MEK1 protein knockdown efficiency of **CLD**/**siMek1** lipoplexs was dose-dependent. When using cationic polymers as the delivery systems, the polymer/nucleic acid mixing ratios are critical for transfection efficiency. The optimal ratio can vary among different formulations and different cell lines.

Discussion

Gene silencing using RNAi demonstrates huge potential in the intervention of many diseases. Due to the poor stability in physiological fluids and inefficient cellular uptake of siRNAs, the delivery of siRNAs into the cytosol of target cells is one of the main challenges in the development of effective siRNA-based therapies. Duplex siRNAs are negatively charged polymers and cannot easily penetrate hydrophobic cellular membranes without assistance. Non-viral vectors, including cationic lipids, polymers, dendrimers and cell-penetration peptides, have been studied for delivering siRNAs because of their lower toxicity, relatively low production cost, high biocompatibility and convenient surface modification.[12] Cationic lipid assisted transfection has shown more success in DNA transfection. For example, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (**DOTMA**) was first reported for use in DNA transfection in the late 1980s.[37] Although commercial agents such as Lipofectin and its new versions, oligofectamine and Lipofectamine RNAiMAX, have already shown satisfactory results for most in vitro applications, they are not suitable for in vivo use because of the toxicity.[38]

The optimal delivery system for clinic use should provide efficient delivery of siRNA into target cells, protect siRNA from rapid emzymatic digestion, and promote the endosomal release into the cytoplasm for gene silencing. They should be biodegradable and have low toxicity too. A number of new cationic lipids have been reported for enhancing the transfection efficiency and also to overcome the problems in pharmacokinetics, toxicity, and immunogenicity of cationic lipids.[32,33] In this study, we synthesized a novel gemini-like cationic lipid (**CLD**) for efficient siRNA delivery and low toxicity. In this new siRNA delivery agent, cystine was used as backbone to link two cationic head groups, lysine at N-terminal, for complex formation with siRNAs and cellular internalization, and to conjugate two hydrophobic chains, oleyl alcohol at C-terminal, for lipid bilayer stability. The redox-sensitive cystine could be reduced inside the cells, thus decreasing the cytotoxicity of cationic lipids.[30]

The **CLD** monomer was synthesized in 5 steps with a total yield of 28.7%. To characterize the properties of the **CLD** liposomes and to assess their applicability for **siMek1** delivery into cells, we

investigated its complexation efficiency and physicochemical properties including size, surface charge, and **CLD**/**siMek1** complex stability. The size of **CLD** liposomes is around 120 nm in diameter, favorable for cellular uptake via endocytosis[39] and able to provide EPR (Enhanced permeability and retention effect) for tumor tissue specific delivery. The positive charges allowed the **CLD** liposomes to form complexes with negative **siMek1** more effectively. The **CLD** liposomes and **siMek1** (50 nM) started to form complexes at the lower charge ratio (N/P) of 6, and the complex formation was complete at the N/P ratio of 12. The polyanions on cell membrane such as sulphated glycosaminoglycans may disrupt the **CLD**/**siMek1** complexes during transfer across the membrane, and reduce **siMek1** delivery efficiency. The heparin displacement assay demonstrated the integrity of the **CLD**/**siMek1** complexes in the presence of polyanions (heparin). Although heparins gradually induced the dissociation of **siMek1** complexes, the **CLD**/**siMek1** complexes were stable even in the presence of 31.3 μg/μL of heparin.

Furthermore, uptake study and gene silencing experiment were carried out in the presence of 10% FBS. The serum stability assay result suggested that the lipoplexes could protect **siMek1** from degradation in the presence of 50% serum solution. In addition, **siMek1** could be released in the presence of the reducing agent **DTT** (Supplementary Fig. S3), mimicking the intracellular reducing environment. This result suggested that the disulfide of **CLD** could be cleaved inside the cells to facilitate intracellular **siMek1** release, which is also in agreement with previous studies.[29,34] Moreover, efficient intracellular release of **siMek1** cargo can positively contribute to the enhanced silencing efficiency. These properties, including low cytotoxicity, strong **siMek1** binding ability and intracellular **siMek1** release, underscore the **siMek1** delivery capability of the **CLD** liposomes, leading to the silencing of MEK1 expression.

The transfections of lipoplexes were demonstrated by the cellular uptake experiment. The HCS results suggested that the cellular uptake of lipoplexes increased with the N/P ratios and showed equal or better efficiency comparing with the commercial reagent, Lipofectamine RNAiMAX. The fluorescence confocal microscopy study demonstrated that the internalized lipoplexes were in the cytoplasm, which showed a punctate pattern. This punctate pattern of fluorescent **siMek1** could suggest that the **CLD**/**siMek1** lipoplexes were uptaken into the cells by endocytotic pathway. Facile escape from the acidic endosomal compartment is necessary for the efficient gene transfer. Our results demonstrated significant gene silencing and protein knockdown, suggesting efficient endosome escape of **siMek1**. This study suggests a new strategy to design biodegradable gemini-like cationic lipid for non-viral delivery of siRNAs.

Conclusion

In conclusion, we have developed a novel gemini-like cationic lipid (**CLD**) containing natural alkaline amino acids with a cystine in the spacer and biocompatible oleyl alcohol for siRNA delivery. It can effectively bind and protect siRNA from degradation. Moreover, the lipoplexes of **CLD**/siRNA can realize efficient cellular uptake and efficient MEK1 gene silencing. Besides, preliminary study for disulfide bridge with reducing reagent DTT has disclosed that cystine linker was biodegradable, and facilitating siRNA endosome escape into cytoplasm. This synthetic biodegradable gemini-like cationic lipid represents a promising carrier for siRNA delivery and would be further investigated widely.

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Notes and references

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‡Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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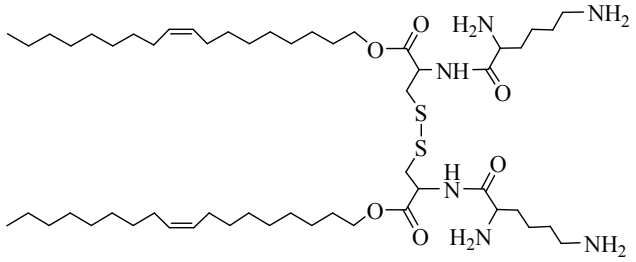
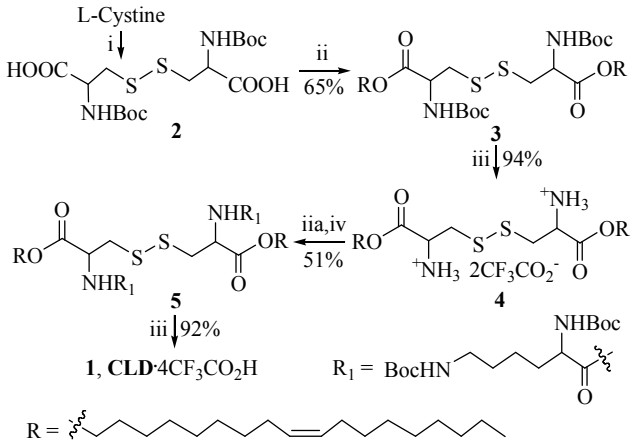


1. CLD

Fig. 1. Structure of gemini-like cationic lipid(**CLD**).



Scheme 1. Synthetic procedure for the preparation of **CLD**. i. Boc_2O, NaOH, H_2O, dioxane, 12h, r.t.; 25 °C, ii. a. DCC, DMAP, CH_2Cl_2; b. Oleyl alcohol(R), r.t., 25 °C; iii. 30% CF_3CO_2H, CH_2Cl_2, r.t., 30 min, iv. Boc-Lys(Boc)-OH(R1).

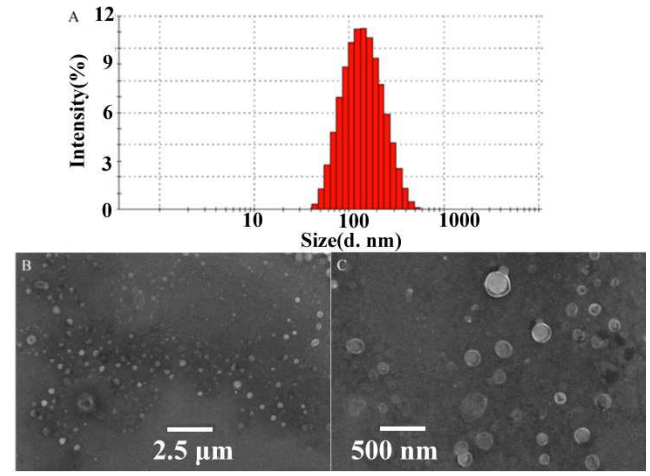


Fig. 2 A. Typical particle size and distribution of **CLD** liposomes, B and C. Scanning electron microscope of **CLD** liposomes.

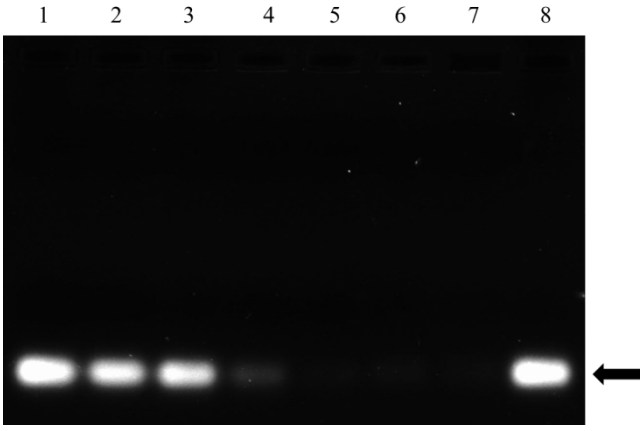


Fig. 3. Electrophoretic retardation analysis of **siMek1** binding by **CLD** liposomes. The lane numbers correspond to different cationic lipid/**siMek1** N/P ratios: 1. 1:1, 2. 2:1, 3. 4:1, 4. 6:1, 5. 8:1, 6. 10:1, 7. 12:1, 8. 0:1 (**siMek1** only). Each lane contains 1.33 μg **siMek1**. The **siMek1** was visualized with Goldenview™ dye staining. The arrows indicated the free **siMek1**.

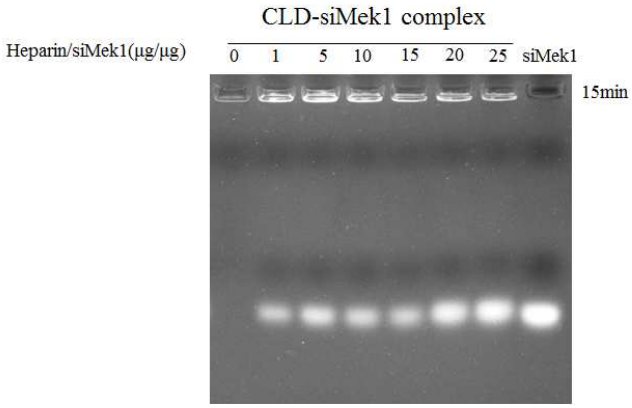


Fig. 4. siMek1 dissociation from complexes by heparin competition. **siMek1** complexed with **CLD** liposomes at a cationic liposomes: siRNA weight ratio of 9:1 (N/P ratio 12) was incubated for 15 min at 37 °C with increasing concentrations of polyanionic heparin. The dissociation was determined by assessing free **siMek1** using agarose gel electrophoresis.

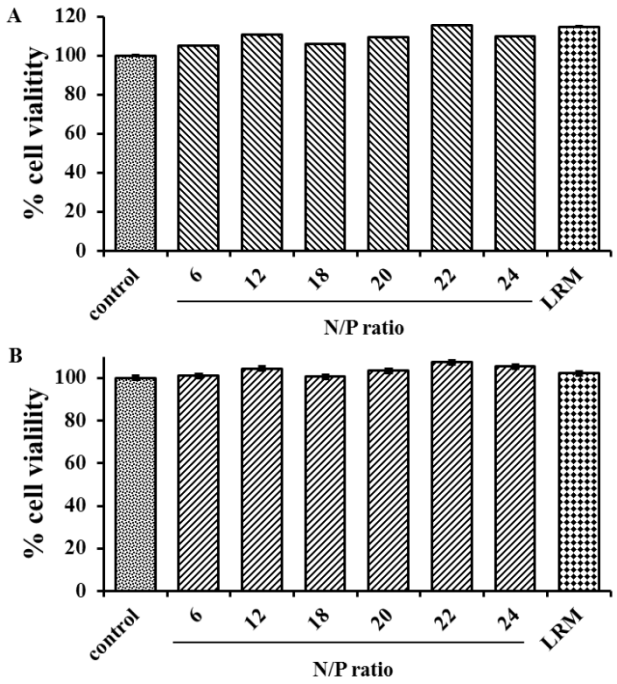


Fig. 5. Cytotoxicity of **CLD** liposomes against HEK293 (A) and HeLa (B) cells. Cells were incubated with different concentrations of **CLD** liposomes (represented by N/P ratios between **CLD** liposomes and **siMek1**, in this experiment no **siMek1** was applied), LRM represented Lipofectamine RNAiMAX, control represented the untreated cells.

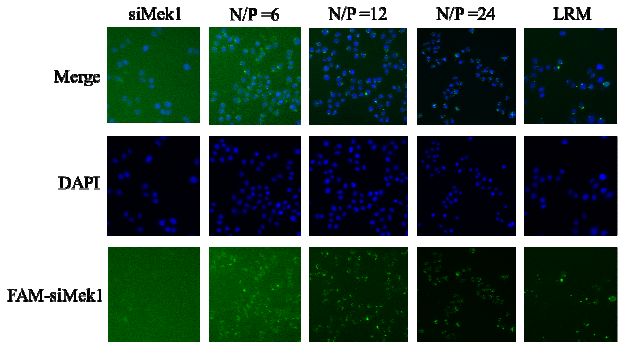


Fig. 6. *In vitro* **siMek1** uptake study by High Content Screening (**HCS**) reader. HeLa cells were transfected with liposomes entrapped with FAM-**siMek1** for 5 h at 37 °C. The final concentration of FAM-**siMek1** was 50 nM. After washing with PBS, the cells were fixed with 4% PFA. Cells were then incubated with **DAPI** for nucleus staining. The fluorescence of the cells was visualized with HCS. Row a: the merged images of **DAPI** and FAM-**siMek1**, Row b: the images of **DAPI** in the cell nuclei, Row c: the images of DAPI in the cytoplasm.

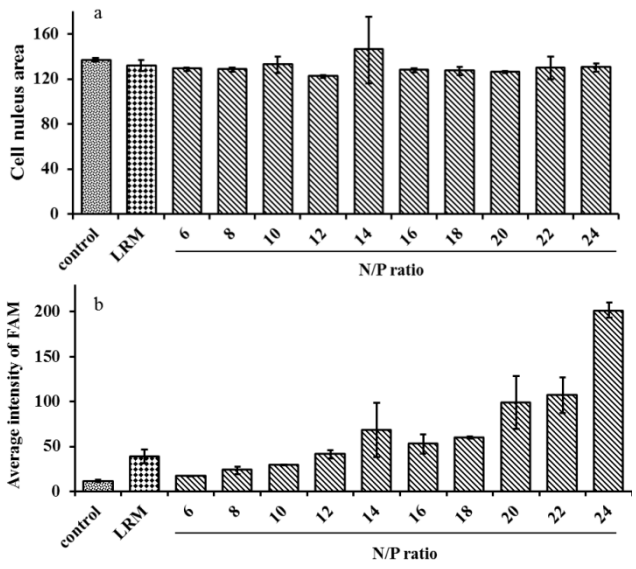


Fig. 7. The average fluorescence intensity of cell nucleus areas by high-content screening assay (**HCA**, a) and the average FAM fluorescence intensity by high-content screening assay (HCA, b). HeLa cells were transfected with liposomes entrapped with FAM-**siMek1** for 5 h at 37 °C. The final concentration of FAM-**siMek1** was 50 nM. After washing with PBS, the cells were fixed with 4% **PFA**. Cells were then incubated with **DAPI** for nucleus staining. The nucleus areas of the cells were visualized with HCS.

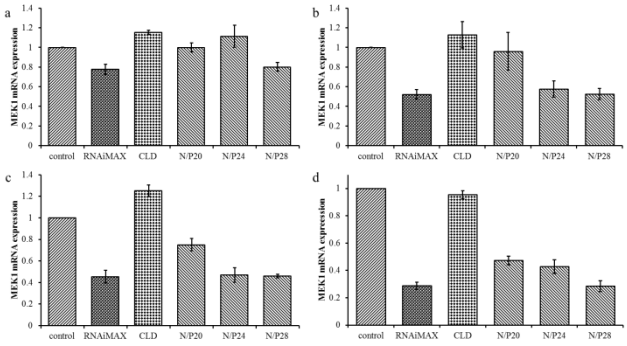


Fig. 8. CLD/siMek1 lipoplexes transfection efficiency at different times (a-d). The gene silencing assay was performed with HeLa cells. Cells were transfected with **siMek1** as lipoplexes at a concentration of 30 nM for 0.5(a), 1(b), 2(c), 4h(d). Afterwards, cells were washed with PBS followed by incubation with **DMEM** for 47.5(a), 47(b), 46(c), 44 h(d). After 48 h, the MEK1 mRNA expression was analyzed, expressed as percent mRNA expression compared to the untreated control. Data are expressed as mean ± standard error for n = 3.

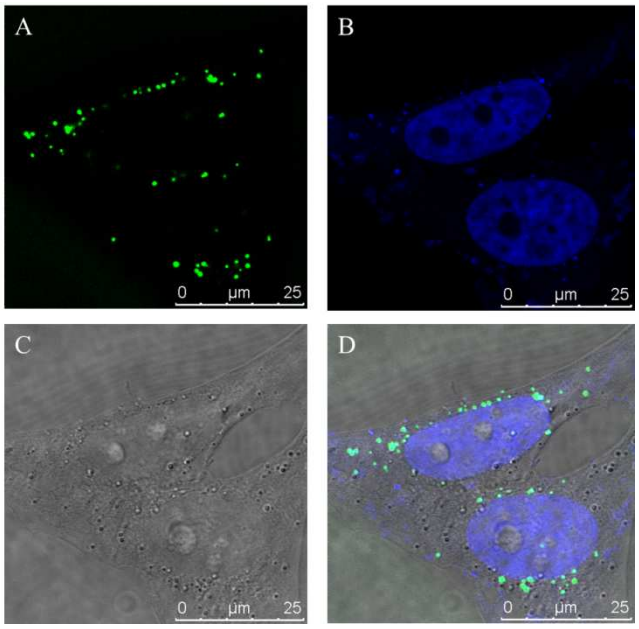


Fig. 9. Confocal microscopy images of HeLa cells (A-D) transfected with liposomes entrapped with FAM-**siMek1** for 5 h at 37 °C. The final concentration of FAM-**siMek1** was 50 nM. Green represents fluorescence of FAM-**siMek1** in the cytoplasm (A). Blue represents fluorescence of **DAPI** in the cell nuclei (B). The phase-contrast of cells (C) and the merged images (D).

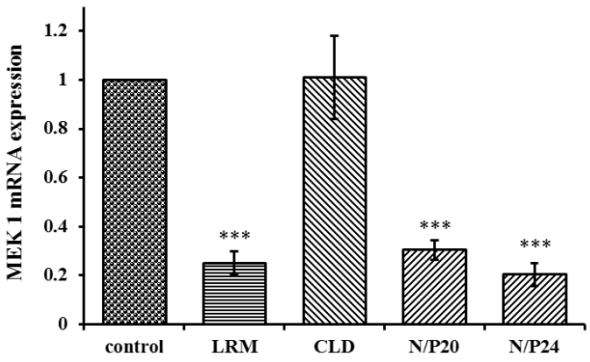


Fig. 10. Gene silencing of **CLD/siMek1** lipoplexes *in vitro*. The gene silencing assay was performed with HeLa cells. After 48 h transfection, the MEK1 mRNA expression was analyzed, expressed as percent mRNA expression compared to the untreated control. Data are expressed as mean ± standard error for n = 3. $^{**}P < 0.01$, $^{***}P < 0.001$.

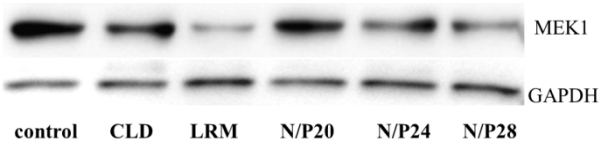


Fig. 11. MEK1 protein expression levels in HeLa cells. Cells were collected at 48h after **siMek1** transfection. The MEK1 protein expression was analyzed, expressed as percent mRNA expression compared to the untreated control. Data are expressed as mean ± standard error for n = 3.