development of new self-assembled cationic amino liposomes

for efficient gene delivery

Yihang Wu,i,ab Yue Xiong,i,ab Ling Wang,i,ab Quanming Zhou,a Linxian Li,b Pavel A. Levkin,b Gary Davidson,*b Liqian Gao* and Wenbin Deng*a

aSchool of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Shenzhen 518107, P.R. China;
bInstitute of Toxicology and Genetics, Karlsruhe Institute of Technology, 76344 Karlsruhe, Germany.
*Corresponding authors: gary.davidson@kit.edu; glqustc@gmail.com or gaolq@mail.sysu.edu.cn; dengwb5@mail.sysu.edu.cn

Table of contents

1. Experimental Section ........................................................................................................... 2

2. Chemicals and Reagents........................................................................................................ 2

3. Apparatus................................................................................................................................ 2

4. Synthesis of Cationic Amino Lipidoids .................................................................................. 3

5. Cationic Liposomal Reagents Preparation ............................................................................. 3

2. Cell Culture and Cell Viability Test ..................................................................................... 3

3. In vitro pDNA Transfection Assay ..................................................................................... 4

4. In vitro siRNA Transfection Assay .................................................................................... 5

5. pDNA Loading Efficiency of A2cT2 .................................................................................... 6

6. Characterization of the Structure of Effective Liposomes ................................................ 7

  6.1 A5cT1.............................................................................................................................. 7

  6.2 A5f2T2............................................................................................................................ 9

  6.3 A2cT2.......................................................................................................................... 11
1. Experimental Sections

1.1 Chemicals and Reagents

N,N-Dimethylmethylenediamine, Tris(2-aminoethyl)amine, 1-(2-Aminoethyl)pyrrolidine, N,N-Diethylethylenediamine, 3-diethylaminopropylamine, 3-Dimethylaminopropylamine, Propargyl Bromide, Propiolic acid, Glycidyl propargyl ether, Allyl bromide, Allyl glycidyl ether, N,N'-Carbonyldiimidazole, Propargyl alcohol, Allylamine, Allyl alcohol, 1-dodecanethiol, 1-Undecanethiol, Ethyl alcohol, 2,2-Dimethoxy-2-phenylacetophenone, were bought from Aladdin. N,N-Dimethylethylenediamine, Propargyl alcohol, Allylamine, Allyl alcohol, were brought from Sigma. 2,2-Dimethoxy-2-phenylacetophenone, Acetic acid, Sodium acetate, EGTA, were brought from Macklin. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was bought from Cordenpharma. Lipofectamine 2000 (Lipo 2000) was purchased from Life Technologies. Dulbecco’s Modified Eagles Medium (DMEM), Phosphate buffer solution (0.1 M, KH$_2$PO$_4$•Na$_2$HPO$_4$, pH 7.4), Fetal bovine serum (FBS), 50×TAE buffer, and Trypsin were bought from Gibco. Agarose was bought from Solarbio.

1.2 Apparatus

Electrophoresis apparatus, purchase from Bio-Rad, is used to evaluate the binding of plasmids to liposomes by electrostatic adsorption. Confocal laser scanning microscope (Leica TCS SPE) and FV1200 Laser Scanning Microscopes which purchase from Olympus is used for High-throughput screening of effective amino cationic liposomes via fluorescence intensity and fluorescence quantity which expressed by cells. BLX-365 is available to provide UV during the synthesis progress. Transmission electron microscope (TEM) image of amino cationic liposomes was observed via a JEOL 2100 microscope operating at 80 kV. Size distribution and zeta
potential of liposomes was performed on a dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS (Malvern).

1.3 Synthesis of Cationic Amino Lipidoids

0.1 mM of a cationic amine and 0.1 mM of an alkyne linker or an alkene linker were combined in a 20 mL glass vial containing 2 mL of Tetrahydrofuran (THF). The glass vial was then shaken at 180 rpm at 40 °C for 20 h. In addition, for CDI based connection of amines and thiols, 0.1 mM of a CDI and 0.1 mM of an alkyne linker or an alkene linker were first combined in a 20 mL glass vial containing 2 mL of DCM and shaken at 180 rpm at room temperature for 1 h. Then 1 mg of DMAP and 0.1 mM of a cationic amine were added into the glass vial and shaken at 180 rpm at room temperature for 20 h. Finally, for all vials, 0.2 mM of an alkyl thiol and 3 mg of photoinitiator (DMPAP) were further added into each glass vial followed by purging with argon. The thiol-yno or thiol-ene radical reaction was then initiated under UV (365 nm, 1.87 mW/cm²) and lasted for 2 h. After evaporation of the solvent, the obtained compound was stored at 4 °C.

1.4 Cationic Liposomal Reagents Preparation

Cationic liposomes were prepared as follows. Cationic amino lipidoids and DOPE were first prepared as 0.01 mol/L ethanol stock solutions. Then the stock solution of amino lipidoids and the stock solution of DOPE were mixed at different molar ratios (1:2, 1:1, 2:1) to produce 300 μL mixtures. 700 μL of 200 mM sodium acetate buffer (pH 5.0) was further added into the mixtures followed by vortexing for 10 s to yield a solution containing 30% ethanol, resulting in the spontaneous formation of liposomes, the final concentration of liposomal reagent for the following transfection experiment is 1 mmol/L.

2. Cell Culture and Cell Viability Test
HEK 293T cells were purchased from the Shanghai Zhong Qiao Xin Zhou Biotech. The cells were cultured using DMEM medium with 10% FBS in a 5% CO\textsubscript{2} incubator at 37 °C. Cell viability was measured using the CCK-8 assay. First, 7.5×10\textsuperscript{3} cells per well were seeded in a 96-well plate and incubated overnight, and then treated with different liposomal reagent and positive control Lipo 2000 under the volume of 0.4 \(\mu\)L combined with 75 ng pDNA per well in 96-well plate. For A2cT2 (the final concentration of working solution is 0.635 mg/mL) and A5f2T2 (the final concentration of working solution is 0.588 mg/mL), detailed volumes (0, 0.1, 0.2, 0.4, 0.8 and 1.6 \(\mu\)L) from working solution were further investigated for the cell viability, compared with positive control Lipo 2000 under the volume of 1.6 \(\mu\)L. After 24 h incubation, 10 \(\mu\)L CCK-8 was added, and the cells were incubated at 37 °C for 1 h with 5% CO\textsubscript{2}. The absorbance at 450 nm was determined and the cell viability was calculated by normalizing average absorbance of the sample wells (n=5) against that of the control wells (without any treatment).

3. In vitro pDNA Transfection Assays

The pDNA (75 ng per well in 96-well plate) and liposomal reagents (0.4 \(\mu\)L per well in 96-well plate) were each diluted in 10 \(\mu\)L of sodium acetate buffer (pH 5.0, 50 mM), combined and then incubated for 30 min at room temperature to allow for lipoplexes formation. For two-step transfection: 80 \(\mu\)L suspension solution of freshly digested HEK 293T cells by trypsin (7.5×10\textsuperscript{3} cells per well in 96-well plate) in DMEM supplemented with 10% FBS was transferred to 96 well plates, and incubated

![Figure S1. The cytotoxicity of A5f2T2, n=5.](image-url)
overnight in a cell incubator. After transfection with lipoplexes, cells were incubated for 20 h at 37 °C and 5% CO₂. pDNA transfection efficiencies of these liposomal reagents were studied by analyzing the fluorescent images obtained by an automatic Olympus IX81 fluorescence microscope. Images were taken automatically in bright field, DAPI, and GFP channels. mESC cells were seeded one day before transfection (1.5×10⁴ per well in 96-well plate). 80 µL of DMEM supplemented with 10% FBS was added to the lipoplexes solution and transferred to 96 well plates. After transfection, cells were incubated for 45 h at 37 °C in 5% CO₂, and then recorded the transfection results by a confocal laser scanning microscope (Leica TCS SPE) with a ×10 objective.

**Figure S2.** (A) The relative transfection efficiency of 83 cationic amine lipidoids library (the molar ratio of lipidoids/DOPE is 1:1). (B) The identity of each lipidoid in our 83-member library for our gene transfection screening.

4. *In vitro* siRNA Transfection Assays

The siRNA (2 pmol per well in 96-well plate) and liposomal reagents (0.2 µL per well in 96-well plate, the molar ratio of lipidoids/DOPE is 1:1) were each diluted in a final volume of 5 µL sodium acetate buffer (pH 5.0, 50 mM), combined and mixed with pipette action to give 10 µL lipoplexes solution and left at room temperature for 30 min. For two-step transfection: U87-Luc cells were seeded one day before transfection (9.0×10³ per well in 96-well plate). Removing the supernatant out of 96-well plates before transfection, and then 80 µL of DMEM supplemented with 10% FBS was added to the lipoplexes solution and transferred to 96-well plates. Cells were
then incubated for 48 h at 37 ºC in 5% CO₂, and then standard luciferase assay was performed and firefly luciferase expression was measured in a luminometer (Perkin Elmer, Victor Light 1420).

Figure S3. The merge layer of the expression of green fluorescent protein (GFP) of U87 Luc cells transfected with the indicated siRNA molecules using A1f2T2 and Lipo 2000 for 48 h, scale bar: 100 μm.

5. pDNA Loading Efficiency of A2cT2

Figure S4. (A) Gel electrophoresis of A2cT2 complexed with pDNA (plasmids with GFP) at varying mass ratios (A2cT2: pDNA). Liposome (L) is able to retard the movement of pDNA through the gel after complexed with pDNA at a ratio of 4:1 when A2cT2 mixed with DOPE
at a ratio of 1:1 or at a ratio of 1:2. (B) By comparing the transfection efficiencies of liposomes mixed in different proportions under different blocking conditions, we found that liposomes show better transfection performance when the plasmids were completely enveloped by liposome.

6. Characterization the Structure of Effective Lipidoids

6.1 A5cT1

Chemical Formula: C$_{37}$H$_{78}$N$_2$O$_2$S$_2$

Exact Mass: 646.55

Mol. Wt.: 647.16

m/e: 646.55 (100.0%), 647.55 (43.6%), 648.55 (10.2%), 648.56 (8.8%), 649.55 (3.9%), 649.56 (1.5%), 647.56 (1.2%)

Elemental Analysis: C, 68.67; H, 12.15; N, 4.33; O, 4.94; S, 9.91
Figure S5. (A) The predictive structure of A5cT1. (B) $^1$H-NMR of A5cT1. $^1$H-NMR (500 MHz, DMSO-d$_6$) $\delta$ 3.99–3.86 (m, 1H), 3.66 (d, $J$=9.6 Hz, 1H), 3.56 (s, 1H), 3.42 (s, 12H), 3.05–2.68 (m, 6H), 2.59–2.52 (m, 3H), 1.99 (s, 2H), 1.51 (s, 4H), 1.22 (d, $J$=22.2 Hz, 42H), 0.86 (s, 6H). (C) $^{13}$C-NMR of A5cT1. $^{13}$C-NMR (500 MHz, DMSO-d$_6$) $\delta$ 73.22, 72.92, 65.72, 50.25, 48.25, 46.91, 45.47, 44.79, 40.16, 39.99, 39.82, 34.38, 32.49, 31.78, 31.05, 29.89, 29.64, 29.50, 29.20, 29.10, 28.66, 22.57, 20.52, 14.41, 8.91. (D) HRMS (ESI$^+$): Calcd for [M$^+$] $^{647.5505}$, Found, 647.5474.
6.2 A5f2T2

Chemical Formula: $C_{33}H_{69}N_{3}O_{2}$

Exact Mass: 587.49

Molecular Weight: 588.06

m/z: 587.49 (100.0%), 588.49 (35.7%), 589.48 (9.0%), 589.49 (3.5%), 590.49 (3.2%), 589.49 (2.7%), 588.49 (1.6%), 588.49 (1.1%)

Elemental Analysis: C, 67.40; H, 11.83; N, 7.15; O, 2.72; S, 10.90
Figure S6. (A) The predictive structure of A5f2T2. (B) \(^1\)H-NMR of A5f2T2, \(^1\)H-NMR (500 MHz, DMSO-d6) \(\delta\) 6.22 (dt, \(J=24.3, 5.9\) Hz, 2H), 3.34–2.98 (m, 11H), 2.83 (t, \(J=6.4\) Hz, 1H), 2.66 (dd, \(J=6.7, 1.8\) Hz, 2H), 2.54 (d, \(J=3.3\) Hz, 3H), 1.77–1.66 (m, 2H), 1.51 (p, \(J=7.2\) Hz, 4H), 1.36–1.29 (m, 4H), 1.22 (d, \(J=26.4\) Hz, 34H), 0.90–0.82 (m, 6H). (C) \(^{13}\)C-NMR of A5f2T2, \(^{13}\)C-NMR (500 MHz, DMSO-d6) \(\delta\) 158.84, 49.00, 46.73, 46.43, 42.94, 39.99, 36.79, 35.20, 32.37, 31.78, 30.48, 29.50, 29.49, 29.45, 29.20, 29.10, 28.68, 25.10, 22.57, 14.42, 8.99. (D) HRMS (ESI\(^+\)): Calcd for \([M^+]\) 588.4882, Found, 588.4879.
6.3 A2cT2

Chemical Formula: C$_{34}$H$_{74}$N$_4$O$_2$S$_2$

Exact Mass: 634.53

Molecular Weight: 635.11

m/z: 634.53 (100.0%), 635.53 (36.8%), 636.52 (9.0%), 636.53 (6.6%), 637.52 (3.3%), 635.52 (1.6%), 635.52 (1.5%)

Elemental Analysis: C, 64.30; H, 11.74; N, 8.82; O, 5.04; S, 10.10
Figure S7. (A) The predictive structure of A2cT2. (B) $^1$H-NMR of A2cT2, $^1$H-NMR (500 MHz, DMSO-d6) $\delta$ 5.14 (s, 1H), 3.69 (d, J=56.7 Hz, 7H), 3.24–2.58 (m, 16H), 2.03–1.89 (m, 2H), 1.50 (s, 4H), 1.25 (s, 38H), 0.86 (t, J=6.5 Hz, 6H). (C) $^{13}$C-NMR of A2cT2, $^{13}$C-NMR (600 MHz, DMSO-d6) $\delta$ 80.82, 77.60, 73.04, 69.63, 58.23, 52.02, 50.95, 45.46, 40.52, 40.40, 40.26, 40.12, 39.99, 39.85, 39.71, 36.80, 35.82, 34.37, 32.47, 31.76, 31.05, 29.89, 29.67, 29.46, 29.20, 28.65, 25.57, 22.57, 14.43. (D) HRMS (ESI$^+$): Calcd for [M$^+$] 635.5253, Found, 635.5215.
7. DLS Measurement

For the study of particle size and surface charge, dynamic light scattering was performed on a Malvern Zetasizer Nano ZS (Malvern). For liposomal and lipoplex samples: liposomes samples (the molar ratio of lipidoids/DOPE is 1:1) were prepared by diluting 32 µL liposomal reagent into 800 µL sodium acetate buffer (50 mM, pH 5.0), and lipoplex samples were prepared by mixing two solutions of 400 µL sodium acetate buffer (50 mM, pH 5.0) which consists of 16 µL liposomes and 30 µL pDNA (100 ng/µL) respectively. Particle size (hydrodynamic diameter) and surface charge (zeta potential) were measured at different time points sequentially by using a standard operating procedure.

8. Transmission Electron Microscopy (TEM)

TEM images were recorded on a JEOL 2100 microscope operating at 80 kV. Samples (the molar ratio of lipidoids/DOPE is 1:1) for TEM were prepared following the method of the delivery gene, and then spreading a drop of cationic liposome which self-assembled in sodium acetate buffer onto standard carbon-coated copper grids (400 mesh). After loading in grids, the samples were stained with 3% Phosphotungstic acid for 5 min, searching negatively stained regions under the transmission electron microscope (TEM).