

## Supporting Information

### Virus-inspired nanovectors: self-assembly of arginine-containing dendritic lipopeptides for improving gene delivery

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# 1 Experimental Section

## 1.1 Materials and methods

2-(1H-Benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl), 1-hydroxybenzotriazole hydrate (HOBT), Boc-Lys(Boc)-OH, Boc-Arg(Pbf)-OH and Boc-Glu-OH were purchased from GL Biochem (Shanghai, China). Branched polyethylenimine (PEI, 25 kDa), propidium iodide (PI), fluorescein isothiocyanate (FITC) and antibiotics (penicillin & streptomycin) were from Sigma-Aldrich (Shanghai, China). (3-Aminopropyl) triethoxysilane, N, N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), thionyl chloride (SOCl<sub>2</sub>) were obtained from Asta Tech Pharmaceutical (Chengdu, China). Methylene chloride (DCM), tetrahydrofuran (THF), dimethyl formamide (DMF), ethyl acetate and n-hexane were purified before use.

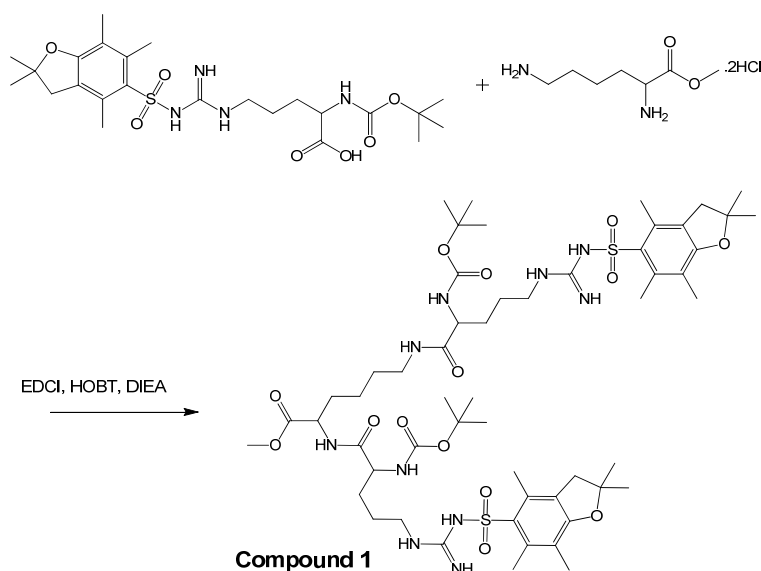
Propidium iodide (PI) and antibiotics (penicillin & streptomycin) were from Sigma-Aldrich (USA). BCA protein assay kit was purchased from Pierce (USA). Cell lysate and the luciferase reporter gene assay kit, pEGFP-C1 (4.7 kb, EGFP encoding plasmid) and pGL3 (5.2 kb, luciferase encoding plasmid) were from Promega (Madison, WI, USA), the plasmids were propagated in *Escherichia coli* DH5 $\alpha$  and extracted using Endo-Free Plasmid Kit (Qiagen, Hilden, Germany). Cell Counting Kit was bought from Dojindo Molecular Technologies (Japan). Nucleic acid labeling kit Label IT®Cy5™ was from Mirus Bio Corporation (USA). The human hepatoma cell line HepG2, murine melanoma cell line B16F10 and human breast cancer cell line MCF7 were obtained from Shanghai Institutes for Biological Sciences (China). HBG buffer (HEPES 20 mM, pH 7.4, 5% glucose) and other buffers were prepared in MilliQ ultrapure water and filtered (0.22  $\mu$ m) before used. All the other chemicals were purchased from Sigma and used as received.

Nanostructures were performed via transmission electron microscope (TEM, JEM-100CX) and atomic force microscope (AFM, MFP-3D-BIO). <sup>1</sup>H-NMR spectra

were recorded on a Bruker Avance II NMR spectrometer at 400 MHz. The molecular weight was tested by Autoflex MALDI-TOF-MS (Bruker, USA). Dynamic light scattering (DLS, Malvern Zetasizer Nano ZS) was used to determine the average particle size of the AVNs and the AVNs/pDNA complex. The cellular uptake were observed by confocal laser scanning microscopy (CLSM, Leica TCP SP5). Microplate Reader (Thermo Fisher Scientific, Varioskan Flash) and inverted fluorescence microscopy (Leica DMI 4000B) were used to detect the cytotoxicity on tumor cells from the quantitative and qualitative analysis. The DNA concentration was determined by UV absorbance at 260 nm.

## 1.2 Synthesis of dendritic lipopeptides

### *Synthesis of dendritic peptide*

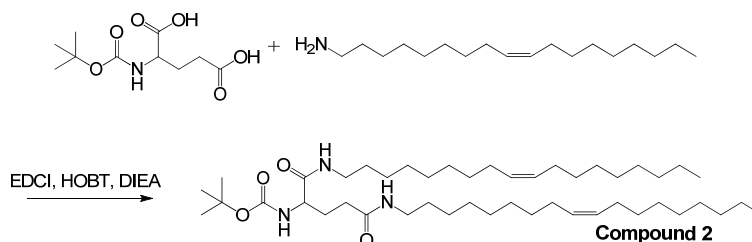


**Scheme S1** Synthetic route of protected dendritic peptide

H-Lys-OMe.2HCl (1.00 g, 4.3 mmol), Boc-Arg(Pbf)-OH (6.78 g, 12.9 mmol), HBTU (4.89 g, 12.9 mmol) and HOBT (1.68 g, 12.9 mmol) were dissolved in anhydrous dichloromethane (DCM, 30 mL) in nitrogen atmosphere (**Scheme S1**). N,N-diisopropylethylamine (DIEA, 5.7 mL, 34.4 mmol) was added in the ice-water bath. This reaction mixture was stirred at room temperature for 2 days. Then, the mixture

was washed with saturated NaHCO<sub>3</sub>, NaHSO<sub>4</sub>, and NaCl solution for several times. The mixture was dried with MgSO<sub>4</sub> for 1 day. After the removal of DCM, the mixture was purified by silica gel column chromatography (DCM/MeOH, 10/1, v/v) to obtain **Compound 1**. (4.41 g, yield: 87%)

### *Synthesis of dual-tail lipids*



**Scheme S2** Synthetic route of protected dual-tail lipids

Boc-Glu-OH (1.00 g, 4.0 mmol), oleylamine (3.20 g, 12.0 mmol), HBTU (4.55 g, 12.0 mmol) and HOBT (1.62 g, 12.0 mmol) were dissolved in anhydrous dichloromethane (DCM, 30 mL) in nitrogen atmosphere (**Scheme S2**). DIEA (2.6 mL, 16.0 mmol) was added in the ice-water bath. This reaction mixture was stirred at room temperature for 2 days. Then, the mixture was washed with saturated NaHCO<sub>3</sub>, NaHSO<sub>4</sub>, and NaCl solution for several times. The mixture was dried with MgSO<sub>4</sub> for 1 day. After the removal of DCM, the mixture was purified by silica gel column chromatography (DCM/MeOH, 10/1, v/v) to obtain **Compound 2**. (2.63 g, yield: 88%)

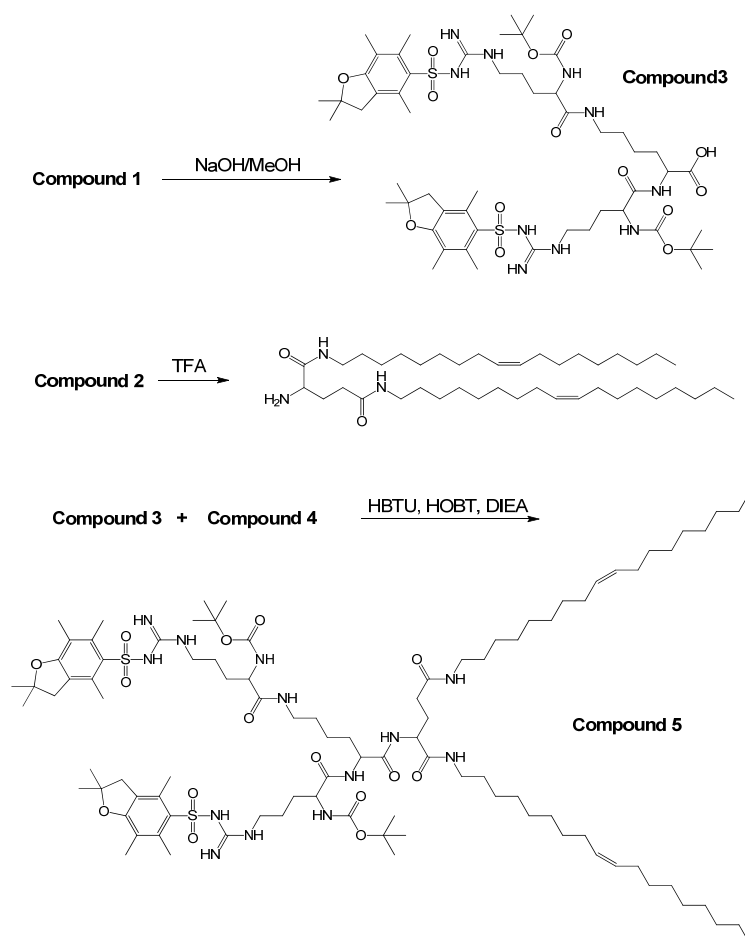
### *Synthesis of dendritic peptide-lipid*

**Compound 1** (4.00 g, 3.4 mmol) was treated with NaOH in 68 mL MeOH (1 mol/L) for 4 hours to expose carboxyl groups. After the removal of MeOH, the mixture was dissolved in H<sub>2</sub>O and adjusted to neutral pH value. **Compound 3** could be extracted by DCM and dried with MgSO<sub>4</sub> for 1 day (2.76 g, yield: 70%).

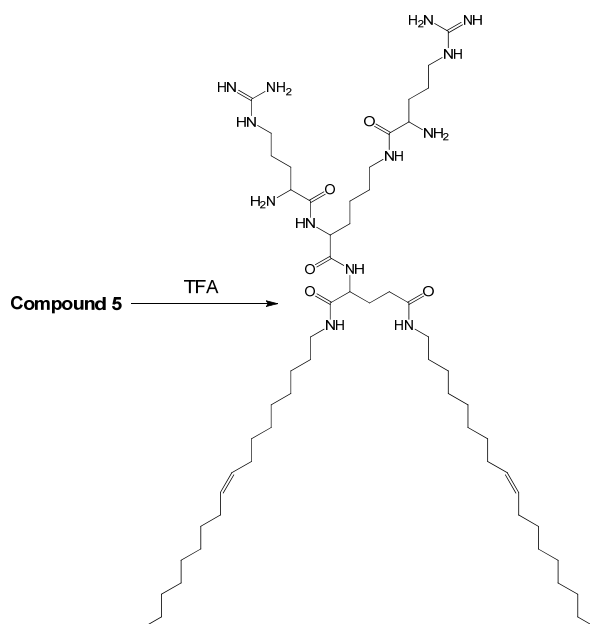
**Compound 2** (2.50 g, 3.4 mmol) was treated with trifluoroacetic acid (2.6 mL, 34 mmol) in nitrogen atmosphere, and the mixture was stirred for 4 hours to put off tert-butyl groups. The mixture was concentrated, and the product was treated with

anhydrous diethyl ether to obtain **Compound 4** (1.98 g, yield: 90%).

**Compound 3** (2.50 g, 2.1 mmol), **Compound 4** (1.63 g, 2.52 mmol), HBTU (1.19 g, 3.15 mmol) and HOBT (0.42 g, 3.15 mmol) were accurately weighted and added into reaction flask. DCM was added into reaction system in nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 30 min after adding DIEA (1.4 mL, 8.4 mmol). This reaction mixture was carried out at room temperature for 2 days. Then, the mixture was washed with saturated NaHCO<sub>3</sub>, NaHSO<sub>4</sub>, and NaCl solution for several times and dried with MgSO<sub>4</sub> for 1 day. After the removal of DCM, the mixture was purified by silica gel column chromatography (DCM/MeOH, 10/1, v/v) to obtain **Compound 5** (3.19 g, yield: 85%).



**Scheme S3** Synthetic route of protected dendritic lipopeptides.



**Scheme S4** Synthetic route of dendritic lipopeptides

In order to expose the amino and guanidine, **Compound 5 (2.03 g, 1.1 mmol)** was treated with trifluoroacetic acid (TFA, 3.4 mL, 44 mmol) in nitrogen atmosphere, and the mixture was stirred for 4 hours to put off protecting groups. After concentration, the product was treated with anhydrous diethyl ether to obtain dendritic lipopeptides. After recrystallization in methanol (100 mL) at 4°C, the dendritic lipopeptides were obtained (yield: 81%).

### 1.3 Preparation and characterization of virus-inspired nanovectors

Dendritic lipopeptides was dissolved in almost 20  $\mu\text{L}$  of methanol, and then injected into 1 mL of HBG buffer (HEPES 20 mM, pH 7.4, 5% glucose) under fast string. After removal of the residual methanol, dendritic lipopeptides would spontaneously self-assemble into virus-inspired nanovectors.

DLS (Malvern Zetasizer Nano ZS) was used to determine the size of virus-inspired nanovectors in PBS (pH = 7.4) at 25 °C. Copper grids were dipped into the fresh SPA solutions to produce the TEM samples. Until the solvent evaporated off, the samples were observed using transmission electron microscopy (TEM, JEM-100CX). After dropping the fresh SPA solution onto the micas, the atomic force microscope (AFM)

samples were dried overnight at room temperature. These samples were observed by AFM (MFP-3D-BIO).

Virus-inspired nanovectors (AVNs) solution was prepared by the methanol injection method. Briefly, dendritic lipopeptides was dissolved in almost 20  $\mu\text{L}$  of methanol, and then injected into 1 mL HBG buffer (HEPES 20 mM, pH 7.4, 5% glucose) under fast string. After removal of the residual methanol, virus-inspired nanovectors were formed spontaneously. AVNs solution was mixed with DNA in HBG buffer at appropriate N/P ratio ranging from 10: 1 to 80: 1. Complex were formed after 20 min incubation at room temperature. The following complex used for transfection and cell apoptosis analysis experiment were formed at N/P ratios of 20:1 and 40:1 unless otherwise specified.

AVNs/DNA complexes were prepared at a final DNA concentration of 3  $\mu\text{g mL}^{-1}$  (total volume of 1 mL) for size distribution measurement, while they were diluted 5-fold in 1 mM KCl solution for zeta potential estimation by Malvern Instruments (Zetasizer Nano ZS, Malvern, UK).

Gel retardation assay was tested for DNA binding ability of complexes. 0.2  $\mu\text{g}$  DNA was mixed with AVNs at different N/P ratios. Ten microliters of complexes solution was loaded onto 0.8% (w/v) agarose gel, electrophoresed with Tris-acetate-EDTA running buffer at 80V for 60 min. DNA on the gel was stained with ethidium bromide, and the result was obtained by Molecular Imager ChemiDoc XRS+ system (Bio-Rad, USA).

## 1.4 Cell culture and cytotoxicity assay

HepG2 cells were preserved in DMEM-HG medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100  $\mu\text{g mL}^{-1}$  streptomycin and 100 IU  $\text{mL}^{-1}$  penicillin. All cell cultures were performed in an incubator with a humidified environment of 5%  $\text{CO}_2$  and a constant temperature of 37°C. The medium was changed twice or thrice a week. Cells were harvested with 0.02 % EDTA and 0.025% trypsin.

The cellular viability of various AVNs/ DNA complexes were evaluated by CCK-8 assay. Cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells per well and cultured overnight. The medium was replaced by 100  $\mu\text{L}$  of fresh medium per well (with or without 10% serum), to which complexes containing 200ng of DNA were added. The PEI/DNA (N/P=10) complex was used as the positive control. 24 h later,

the medium was again replaced by 100  $\mu$ L of fresh medium containing 10% CCK-8 for another 2 h- incubation at the same incubation conditions. After that, the absorbance of solution was read by a microplate reader (Bio-Rad, Model 550, USA) at 450 nm. The cytotoxicity of the complexes was expressed as a relative to untreated cell controls taken as 100% metabolic activity.

### **1.5 *In vitro* transfection of pEGFP and pGL3 plasmid**

Cells (HepG2, MCF7, B16F10) were seeded in 24-well plate at a density of  $4 \times 10^4$  per wells (or 96-well plate at a density of  $1 \times 10^4$  cells per wells) in 100  $\mu$ L of 10% FBS containing cell culture medium and grown to reach 70 - 80% confluence. Prior to transfection, the medium was replaced with 100  $\mu$ L of fresh medium with or without 10% serum, to which various complexes (PEI/DNA and AVNs/DNA) were added with the same amount of 800 ng (or 200 ng) pEGFP or pGL3 plasmid DNA per well. After 4-h incubation, the transfection medium was refreshed, and the cells were grown for an additional incubation before they were processed for expression analysis. Qualitative evaluation of pEGFP transfection was observed 48 h later by an inverted fluorescence microscope (Leica, Germany). Quantitative measurement of luciferase assay was performed 24 h post transfection according to manufacturer's protocols. Relative light units (RLU) was measured by the luciferase reporter gene assay kit on a microplate reader (Bio-Rad, Model 550, USA) and the protein content of the lysed cell was determined by BCA protein assay. Luciferase activity was expressed as the relative fluorescence intensity per mg protein (RLU/mg protein).

### **1.6 Cell apoptosis analysis**

Ten thousands of HepG2 cells were seeded in 96 wells plate and cultured until confluency reaching 60 - 80%. Cells were incubated with various complexes containing 200 ng pGL<sub>3</sub> plasmid in the absence or presence of 10% FBS for 4 h. After the media was replaced, the cells were incubated for an additional 20 h, and washed with PBS. 100  $\mu$ L of 10  $\mu$ g mL<sup>-1</sup> propidium iodide (PI) was added into each well and placed in the incubator for 15 min. The cells were then washed with PBS and cultured in fresh DMEM before observation by inverted fluorescence microscope (Leica CTR 4000, Wetzlar, Germany).



## 1.7 Confocal laser scanning microscope and flow cytometry

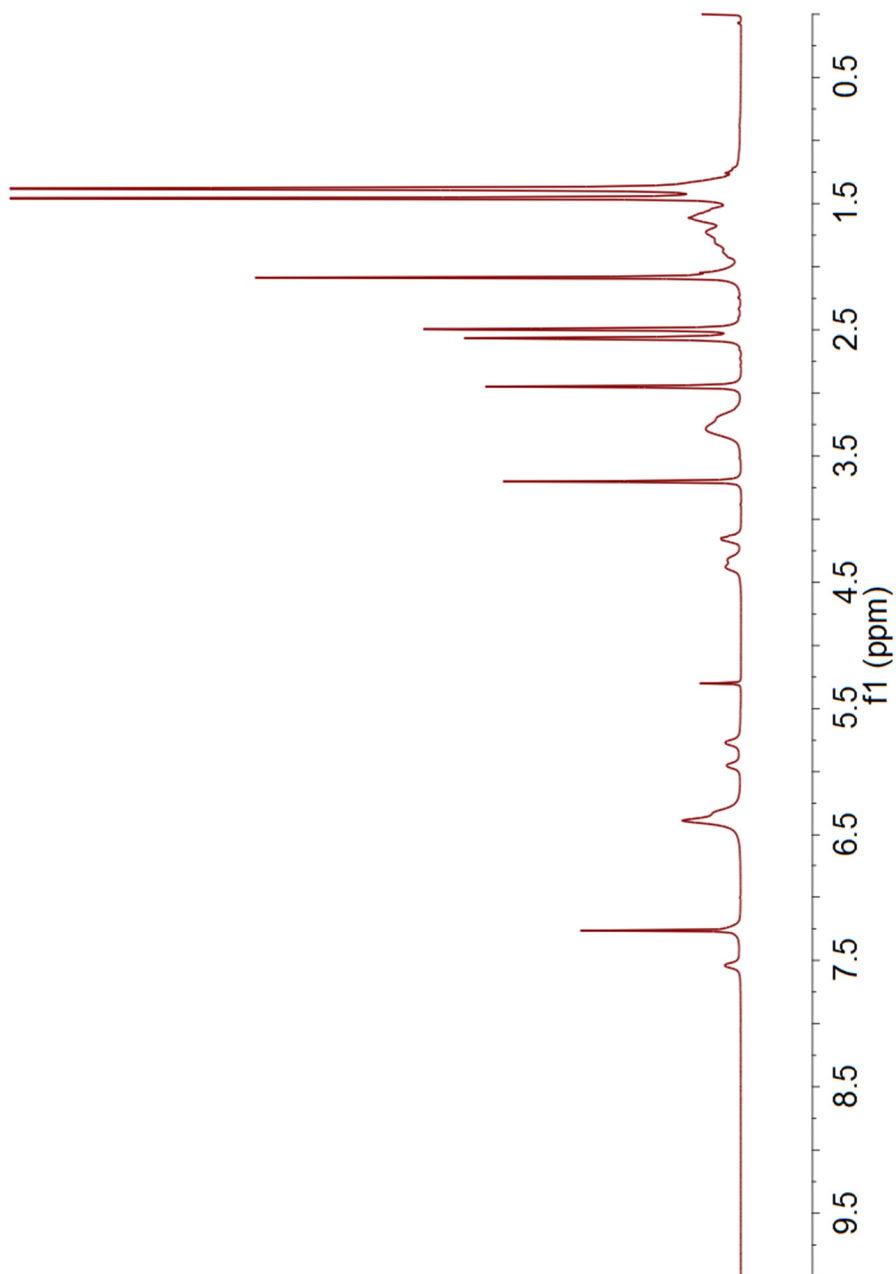
pGL3 plasmid was labeled with Cy5 by the Label IT® Kit for intuitive trafficking the movement process of DNA. According to manufacturer's specifications, the average density of fluorescent dyes is one dye molecule per 380 DNA base pairs. While the cationic dendritic peptide-lipid and PEI were labeled with green fluorescent fluorescein isothiocyanate dye (FITC). As described previously, FITC was mixed with equal molar amount of cationic lipid or PEI in DMF under argon atmosphere for 2 h. After reaction, the free fluorescent dyes were removed by dialysis (MWCO 3000Da), and the labelled lipid and PEI was collected by lyophilization. HepG2 cells were seeded at a density of  $2 \times 10^4$  cells per well in  $35 \times 12$  mm glass-bottomed chamber (NEST, China). After overnight culture, the medium was replaced with fresh medium containing 20% Cy5-labeled DNA and 5% FITC-labeled vectors (300 ng DNA per well). At every desired time point (1.5, 2.5 h), the cells were washed twice with PBS (pH 7.4) and fixed with 4% formaldehyde. Microscopical analysis was performed with a confocal laser scanning microscope (CLSM, Leica TCS SP5) equipped at 488 and 633 nm emitted by an argon and two helium/neon lasers delivering light, respectively. Light was collected through a  $63 \times 1.4$  NA oil immersion objective. The Cy5 fluorophore was excited at 543 nm and the emission range was from 565 nm to 615 nm, while FITC was excited at 495 nm and the emission range was from 515 nm to 545 nm. Analytical software was LAS AF Lite 2.3 software (Leica, Germany). The quantified cellular uptake was detected using a fluorescence-activated cell sorting (FACS, Beckman Coulter Cytomics FC-500, USA) with  $1 \times 10^4$  cells.

## 1.8 Statistical analysis

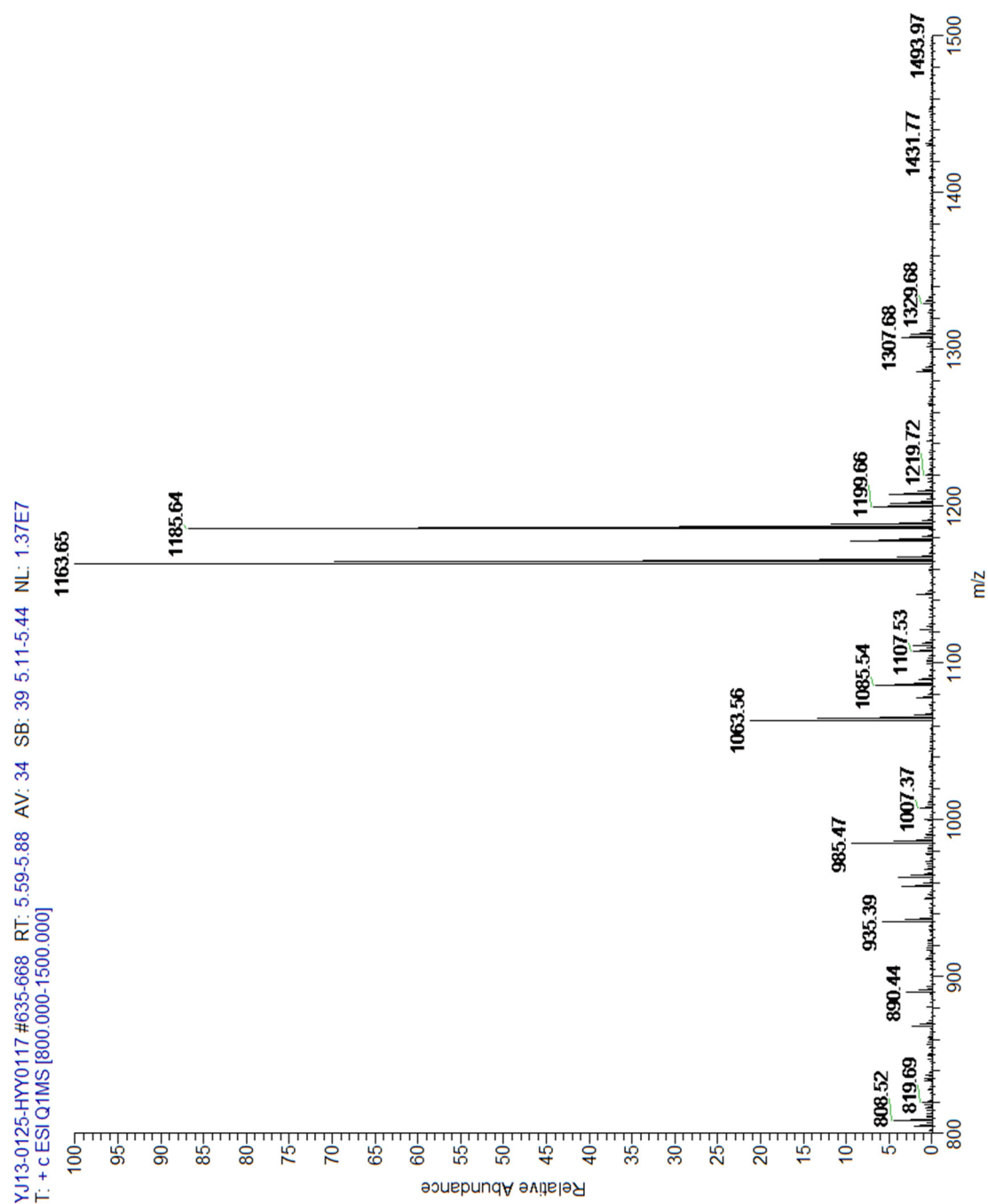
All data represented the mean out of at least five independent measurements and each measurement was performed in triplicate. Data are given as mean values  $\pm$  S.D. Statistical significance ( $p < 0.05$ ) was evaluated by using Student t-test when only two groups were compared. While Differences between treatments groups (more than two groups) were determined by two-way ANOVA. In all tests, a p value less than 0.05 was considered statistically significant.

## 2 Supporting Results

### 2.1 Characterizations of dendritic peptide

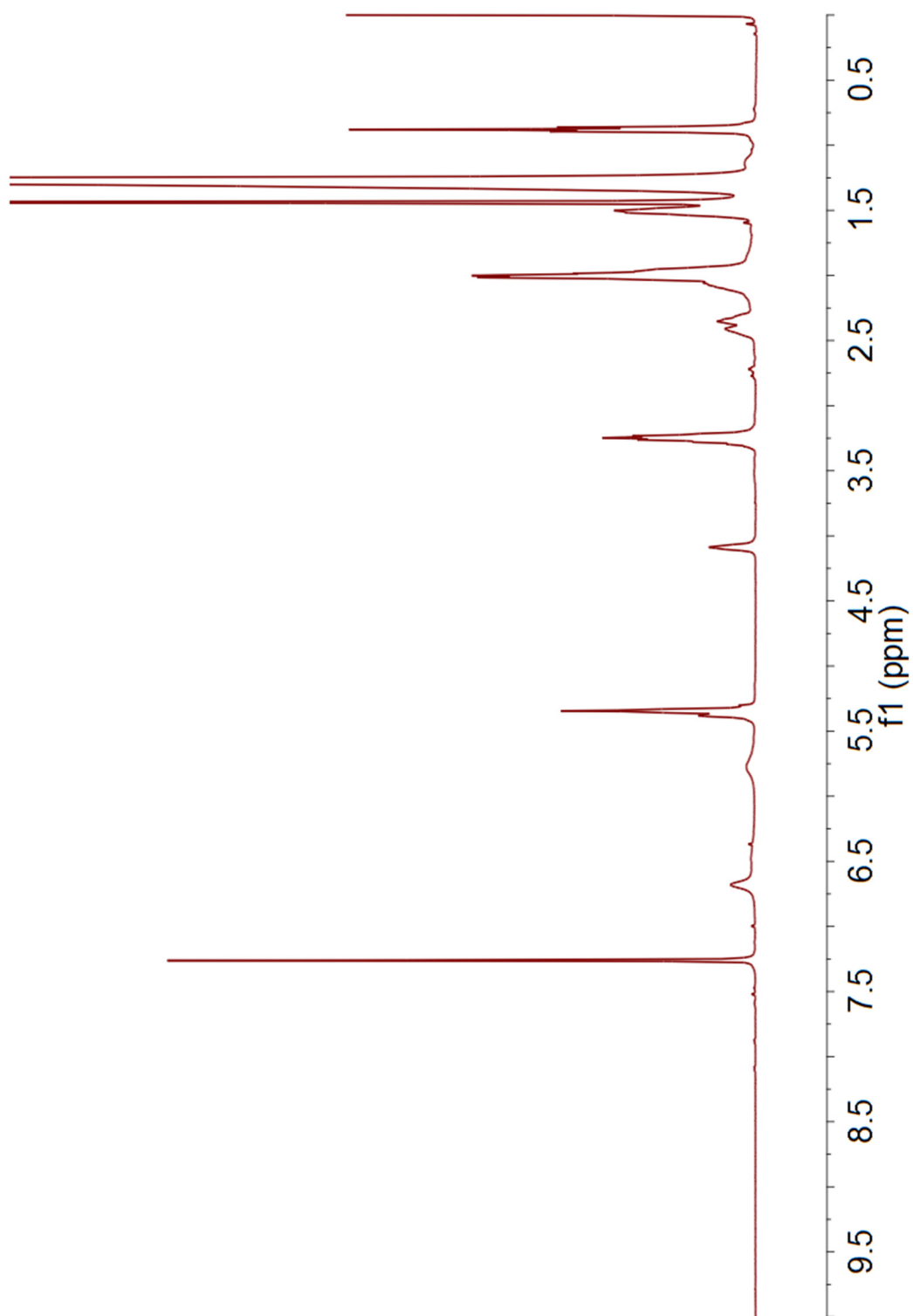


**Figure S1**  $^1\text{H}$  NMR spectrum of protected dendritic peptide (**Compound 1**) in  $\text{CDCl}_3$ .

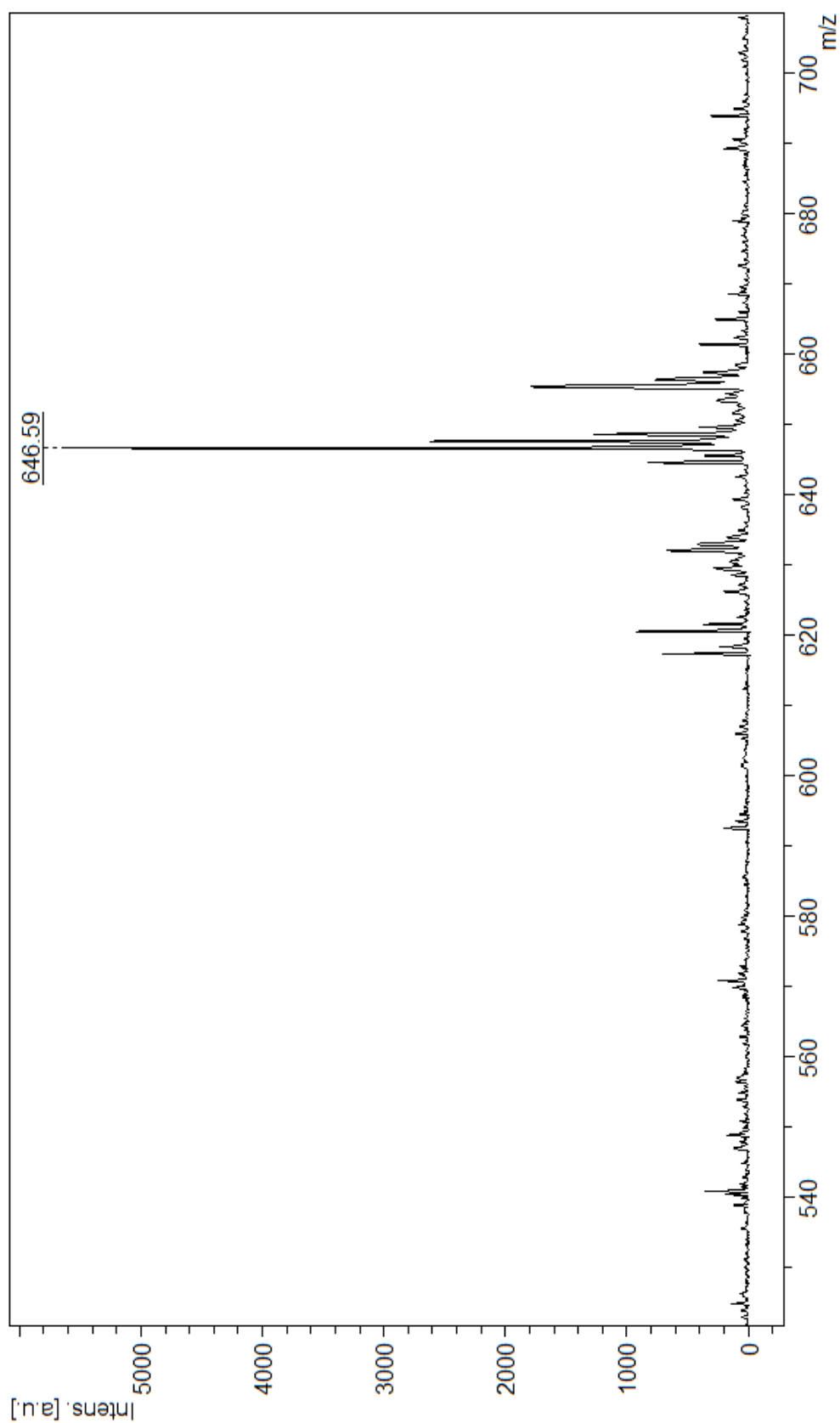


**Figure S2** ESI-MS of dendritic peptide (Compound 3).

## 2.2 Characterizations of dual-tail lipids

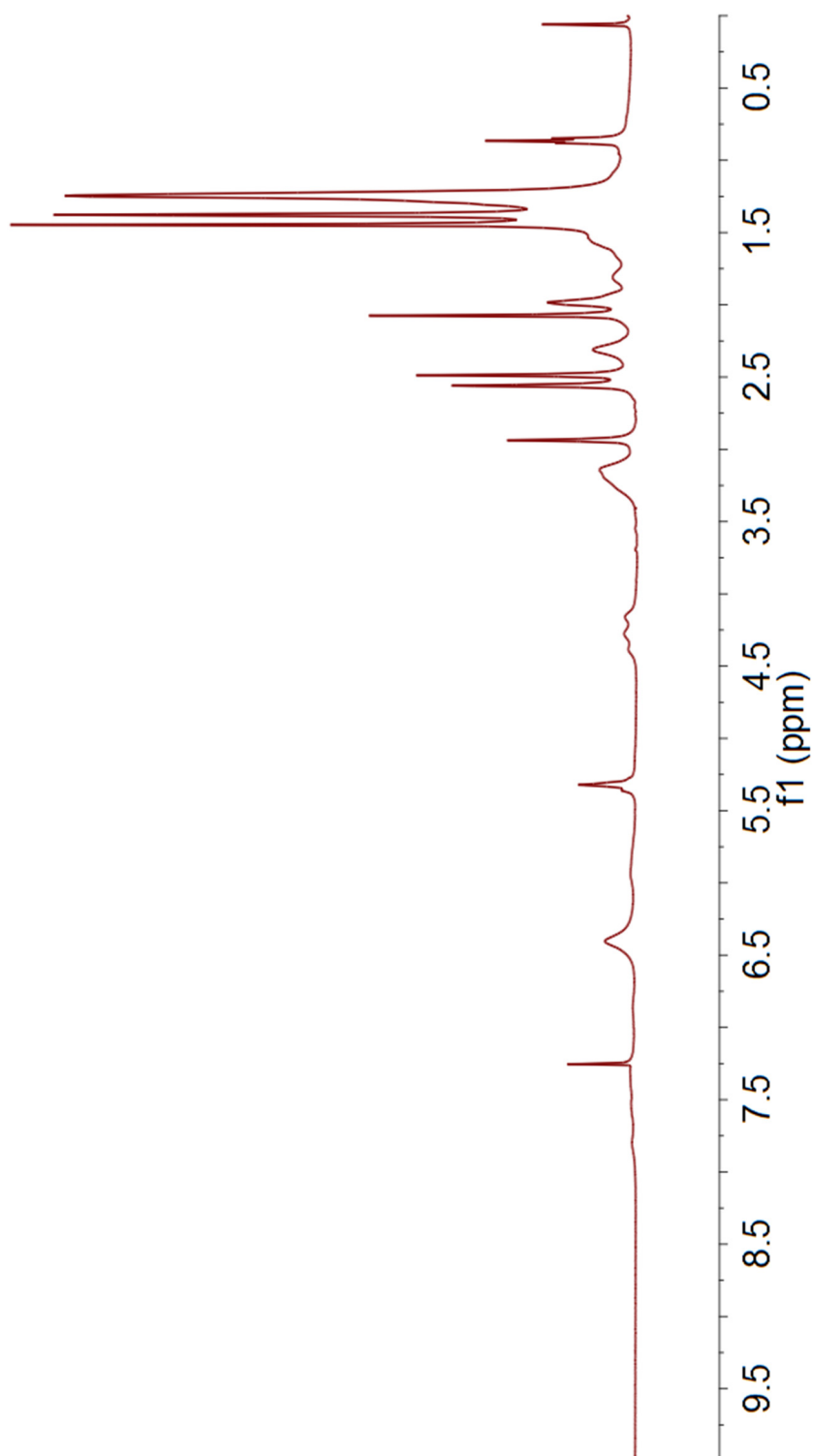


**Figure S3**  $^1\text{H}$  NMR spectrum of protected dual-tail lipids (**Compound 2**) in  $\text{CDCl}_3$ .

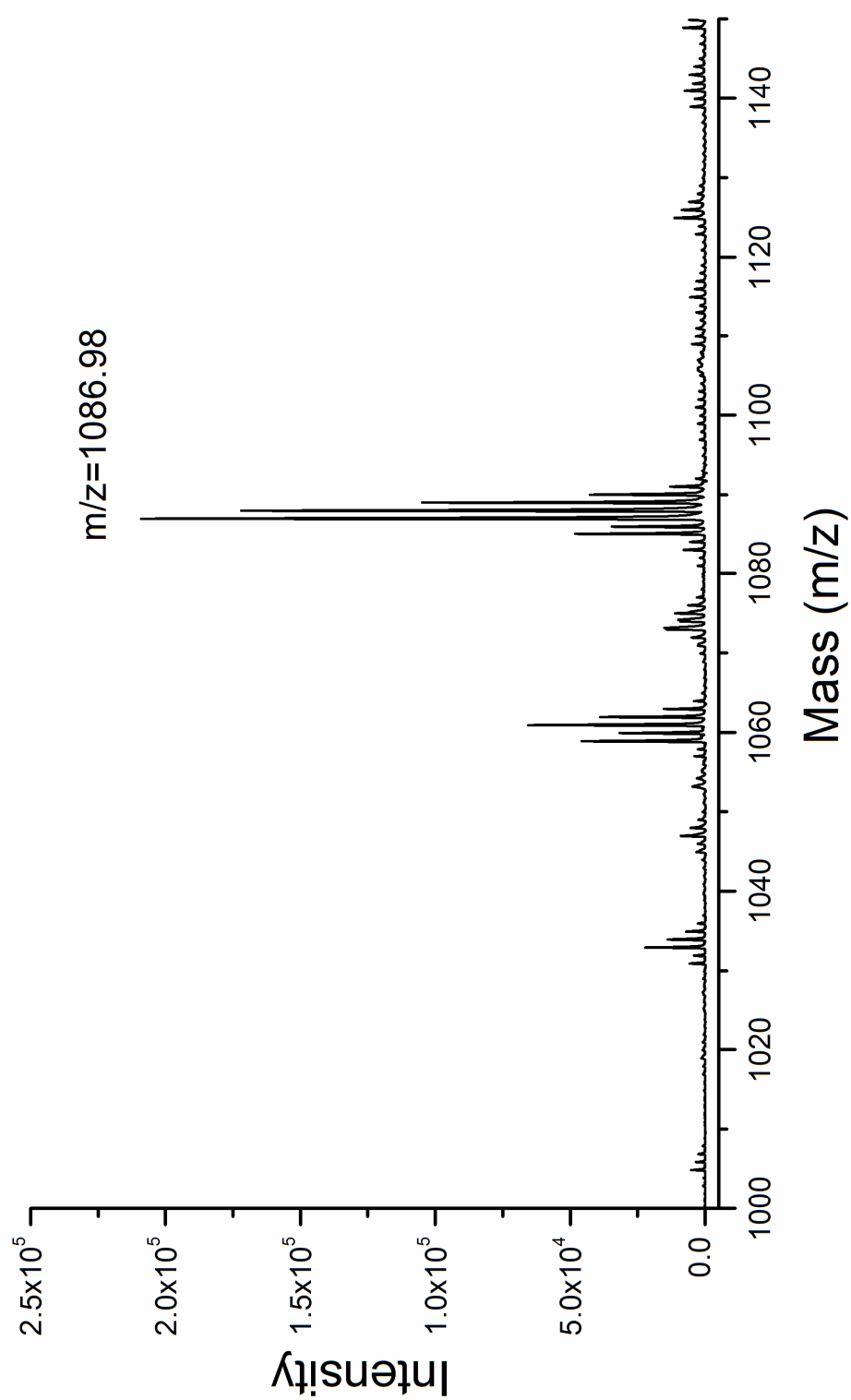


**Figure S4** ESI-MS of dual-tail lipids (**Compound 4**).

## 2.3 Characterizations of dendritic lipid

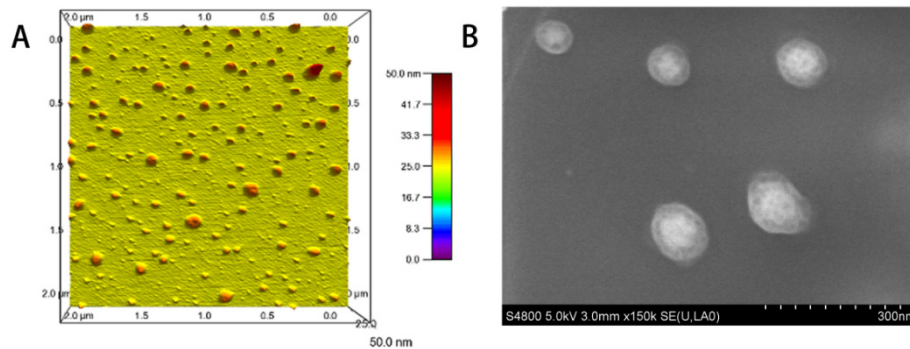


**Figure S5**  $^1\text{H}$  NMR spectrum of protected dendritic lipopeptides (**Compound 5**) in  $\text{CDCl}_3$ .



**Figure S6** MALDI-TOF MS spectrum of dendritic lipopeptides (**Compound 5**).

## 2.4 Characterizations of virus-inspired nanovectors



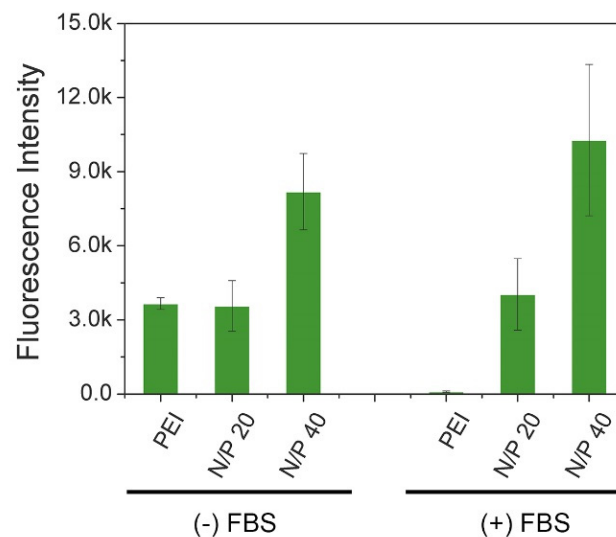
**Figure S7** (A) AFM image and (B) SEM image of virus-inspired nanovectors.

**Table 1.** Size and zeta potentials of DNA-packaged AVNs with different N/P ratios.

Group	N/P 10	N/P 20	N/P 40
Size <sup>(a)</sup>	136.1 nm	107.0 nm	68.0 nm
Zeta Potential <sup>(a)</sup>	35.7 mV	41.5 mV	47.0 mV

(a) average value, n = 3.

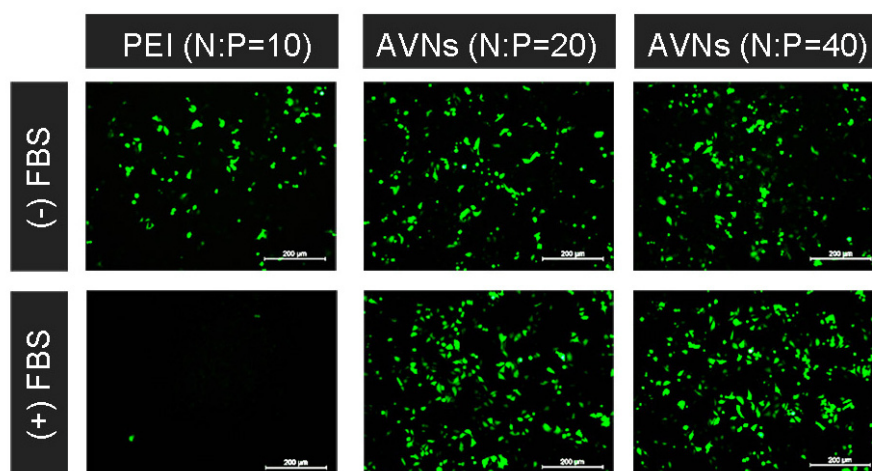
## 2.5 *In vitro* transfection in HepG2



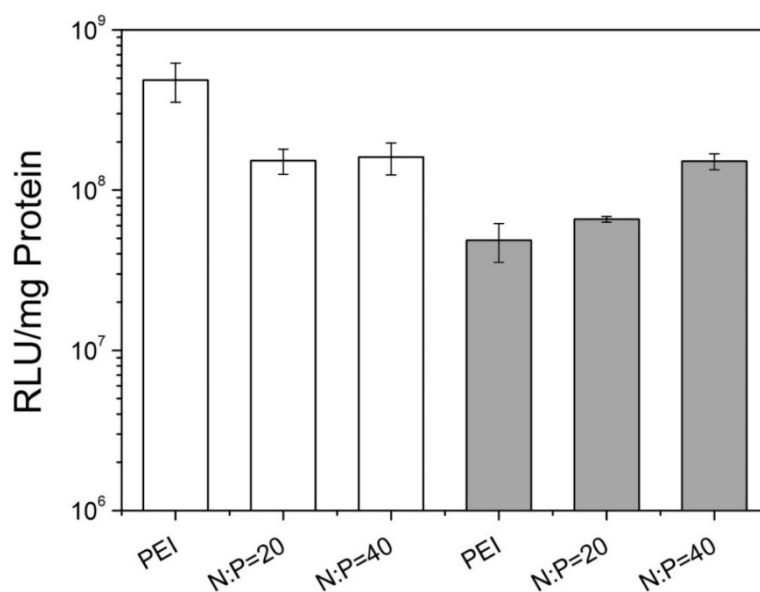
**Figure S8** Green fluorescence intensity of GFP expression in HepG2 cells after exposure to PEI/pEGFP-C1 complex or AVNs/pEGFP-C1 complex with and without FBS for 48 hours (n=3).



## 2.6 *In vitro* transfection in MCF7

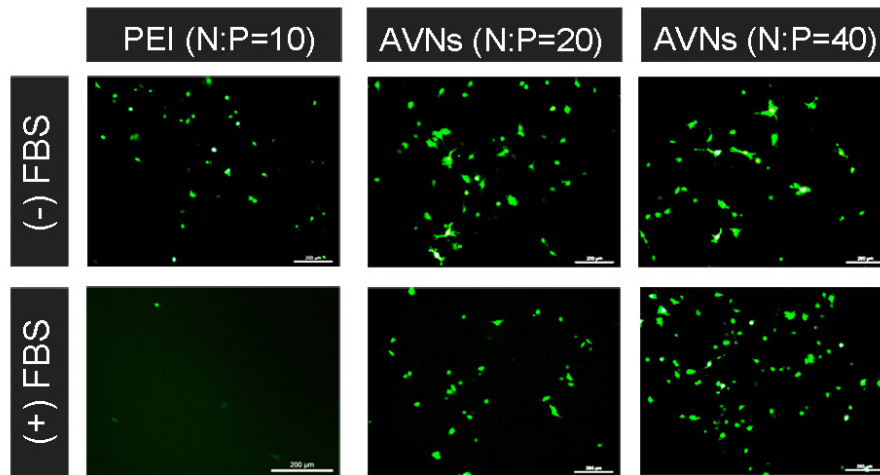


**Figure S9** GFP expression in MCF7 cells after exposure to PEI/pEGFP-C1 complex or AVNs/pEGFP-C1 complex with and without FBS for 48 hours.

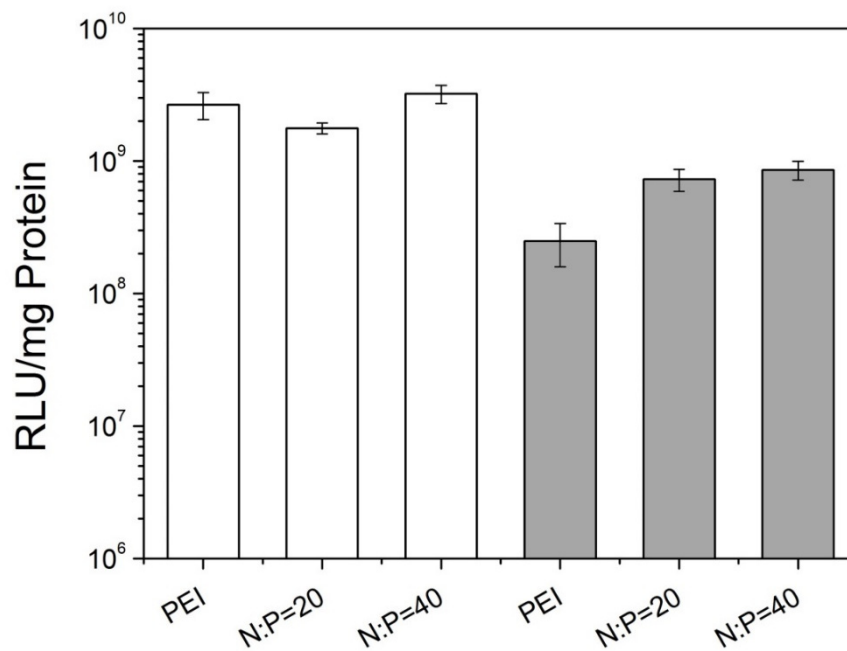


**Figure S10** Luciferase gene transfection in MCF7 cells after exposure to PEI/pGL3-Luc complex or AVNs/pGL3-Luc complex without (white) and with (grey) FBS for 48 hours (n=6).

## 2.7 *In vitro* transfection in B16F10

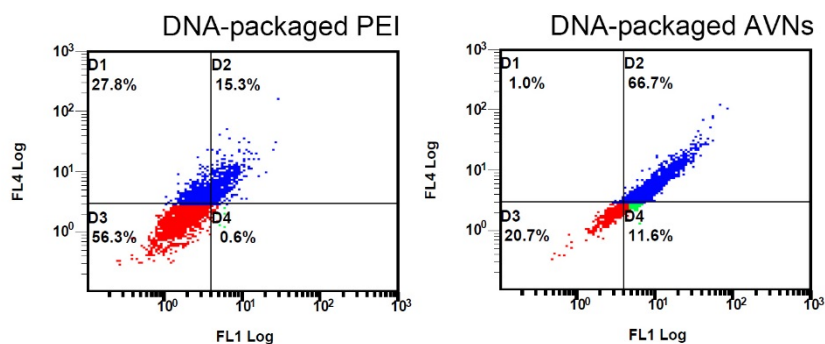


**Figure S11** GFP expression in B16F10 cells after exposure to PEI/pEGFP-C1 complex or AVNs/pEGFP-C1 complex with and without FBS for 48 hours.



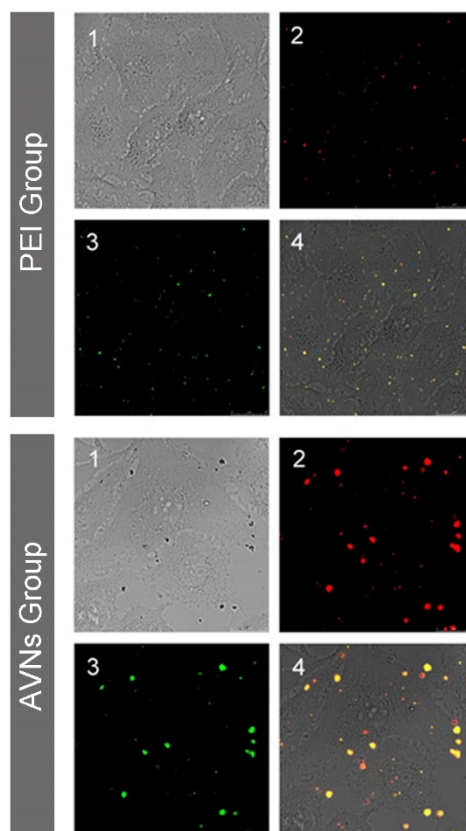
**Figure S12** Luciferase gene transfection in B16F10 cells after exposure to PEI/pGL3-Luc complex or AVNs/pGL3-Luc complex without (white) and with (grey) FBS for 48 hours (n = 6).

## 2.8 Fluorescence-activated cell sorting analysis

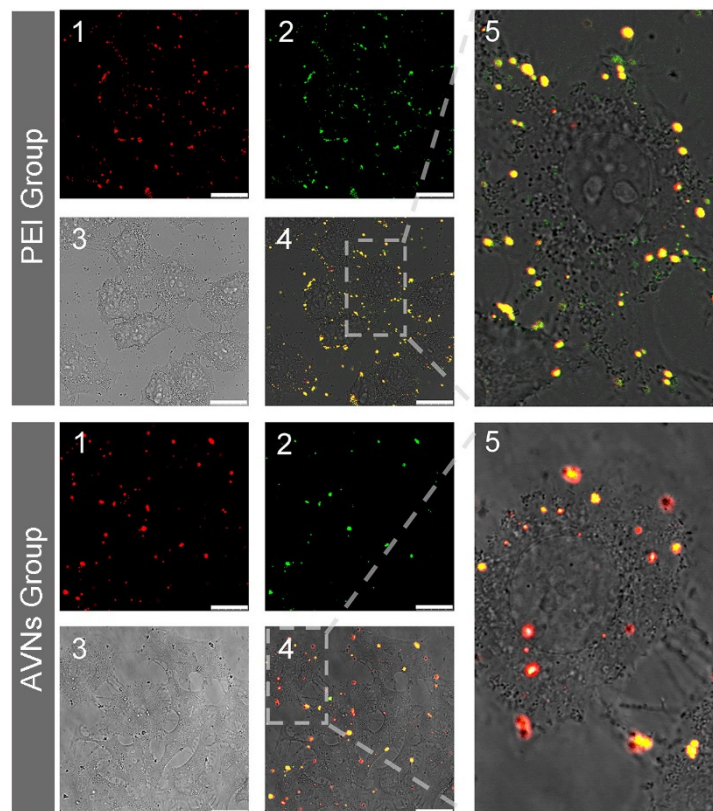


**Figure S13** Flow cytometry analysis of HepG2 cells after incubation with fluorescence labelled DNA-packaged PEI and DNA-packaged AVNs for 1.5 h without FBS.

## 2.9 CLSM images for intracellular delivery



**Figure S14** CLSM images for intracellular delivery of the fluorescence labelled DNA-packaged PEI and DNA-packaged AVNs in HepG2 cells for 1.5 h, including the bright field (1), the Cy5 channel (2), the FITC channel (3) and overlay of the Cy5 channel, FITC channel and bright field (4).



**Figure S15 (A)** CLSM images for intracellular delivery of the fluorescence labelled DNA-packaged PEI and DNA-packaged AVNs in HepG2 cells for 1.5 h, including the bright field (1), the Cy5 channel (2), the FITC channel (3) and overlay of the Cy5 channel, FITC channel and bright field (4).