Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

A biodegradable amphiphilic poly(aminoester) dendrimer for safe and effective siRNA delivery

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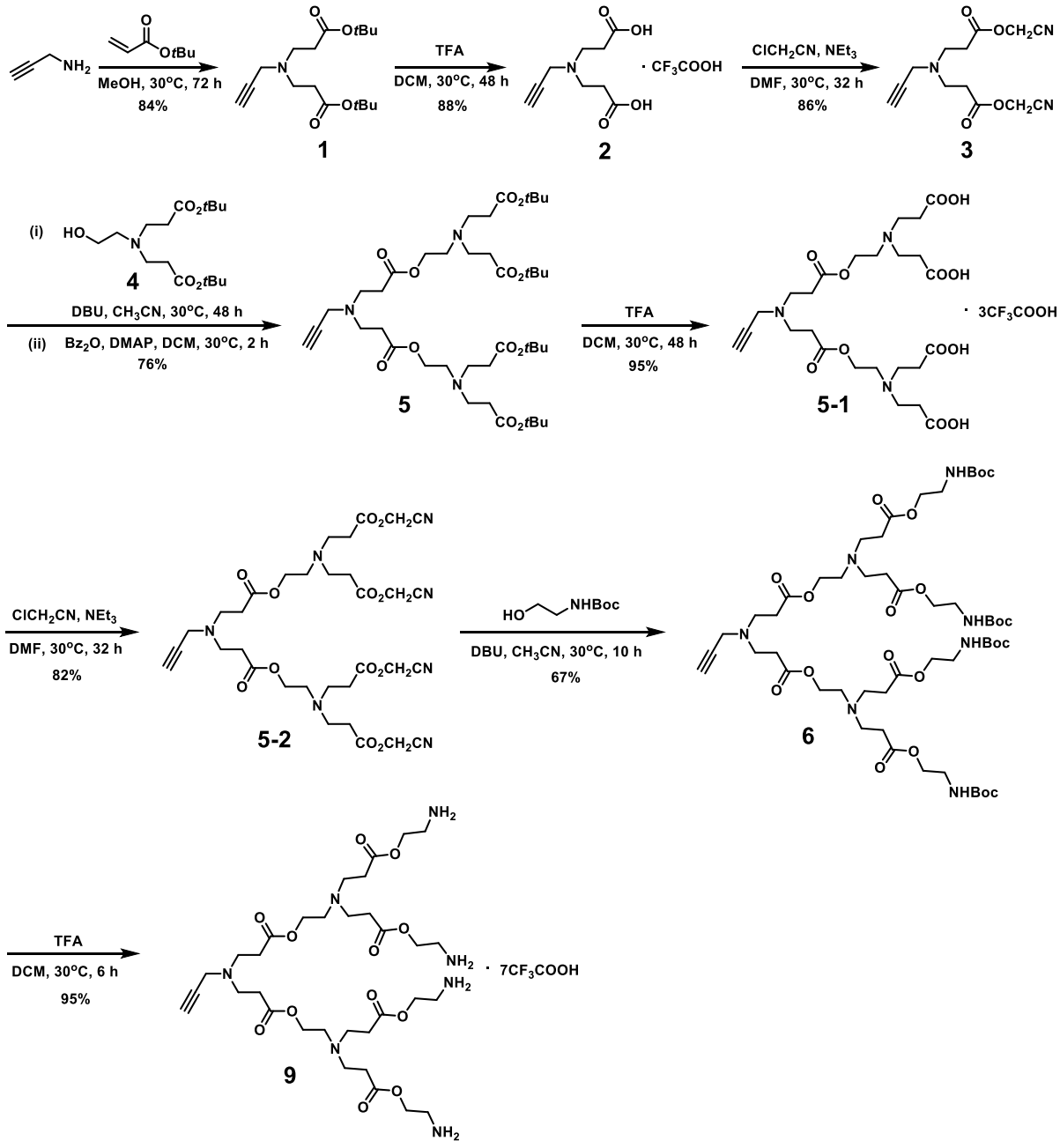
Scheme S1 Synthetic route of the hydrophilic dendron.



Scheme S2 Synthetic route of the hydrophobic part.

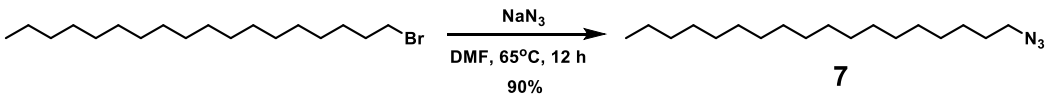
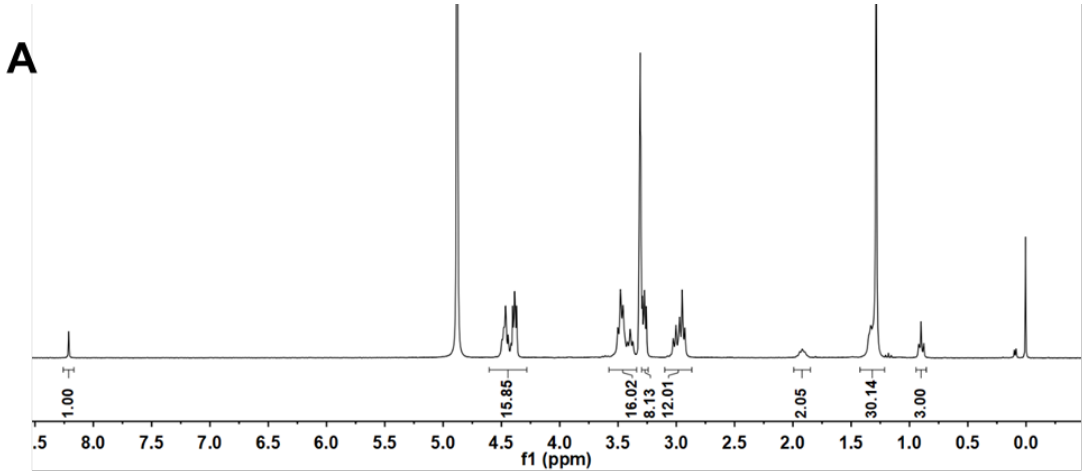
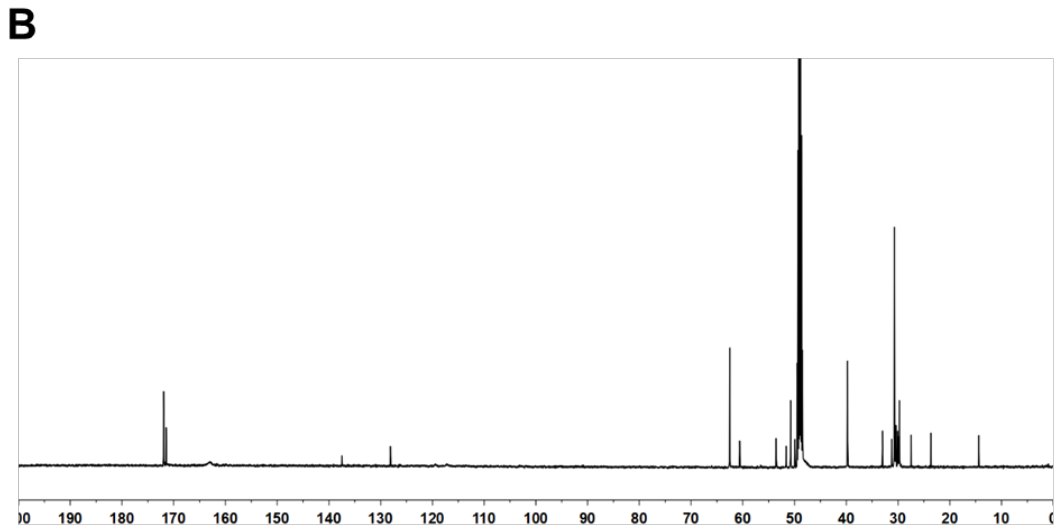


Fig. S1 (A)^{1}H NMR, (B)^{13}C NMR and (C) high-resolution mass spectrometry (HRMS) characterization of **I**





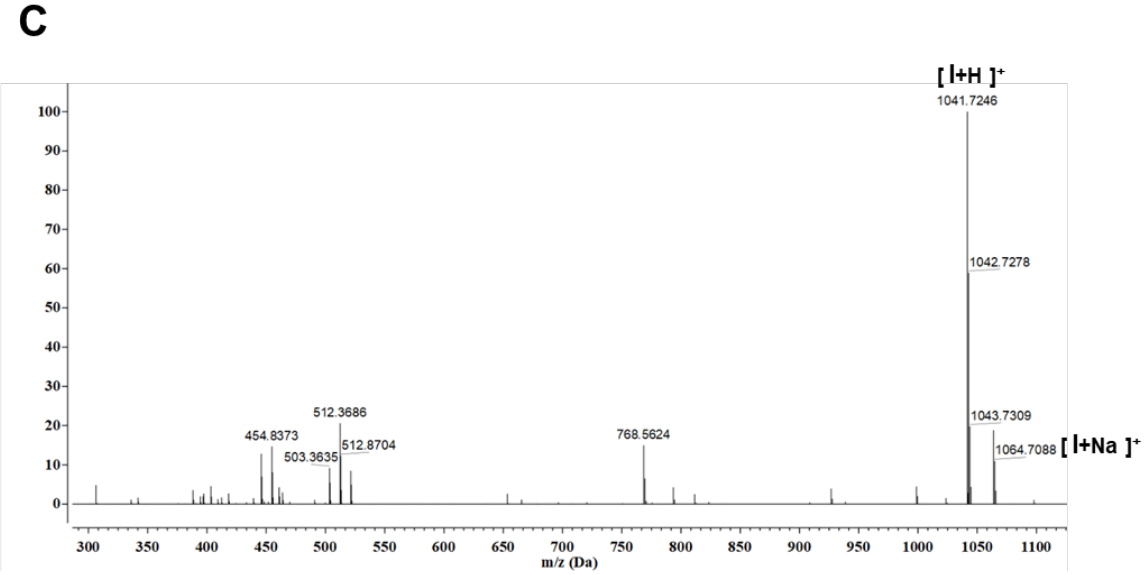
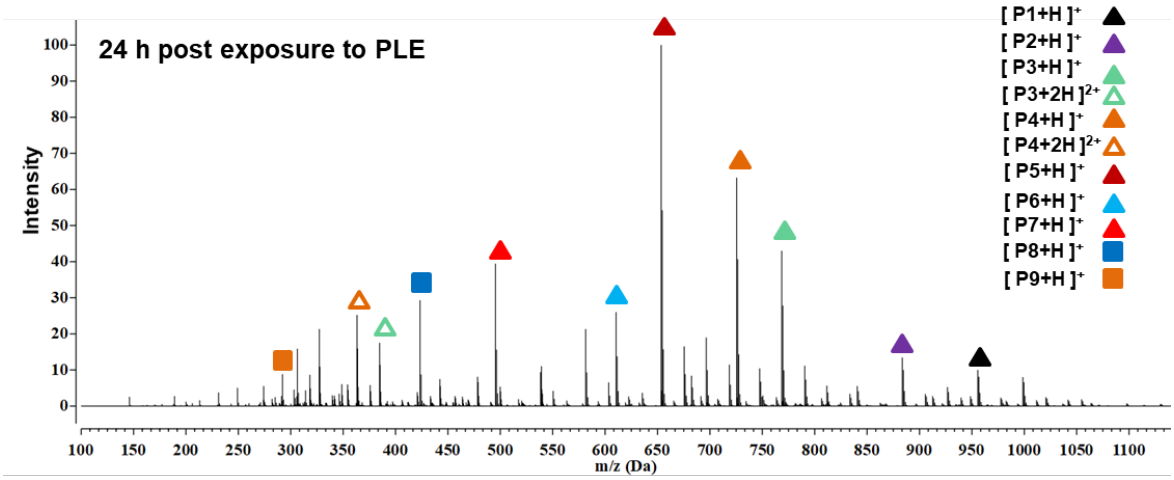


Fig. S2 Biodegradation of **I** in the presence of esterase PLE. HRMS of **I** recorded after exposure to PLE and representative degradation products identified.



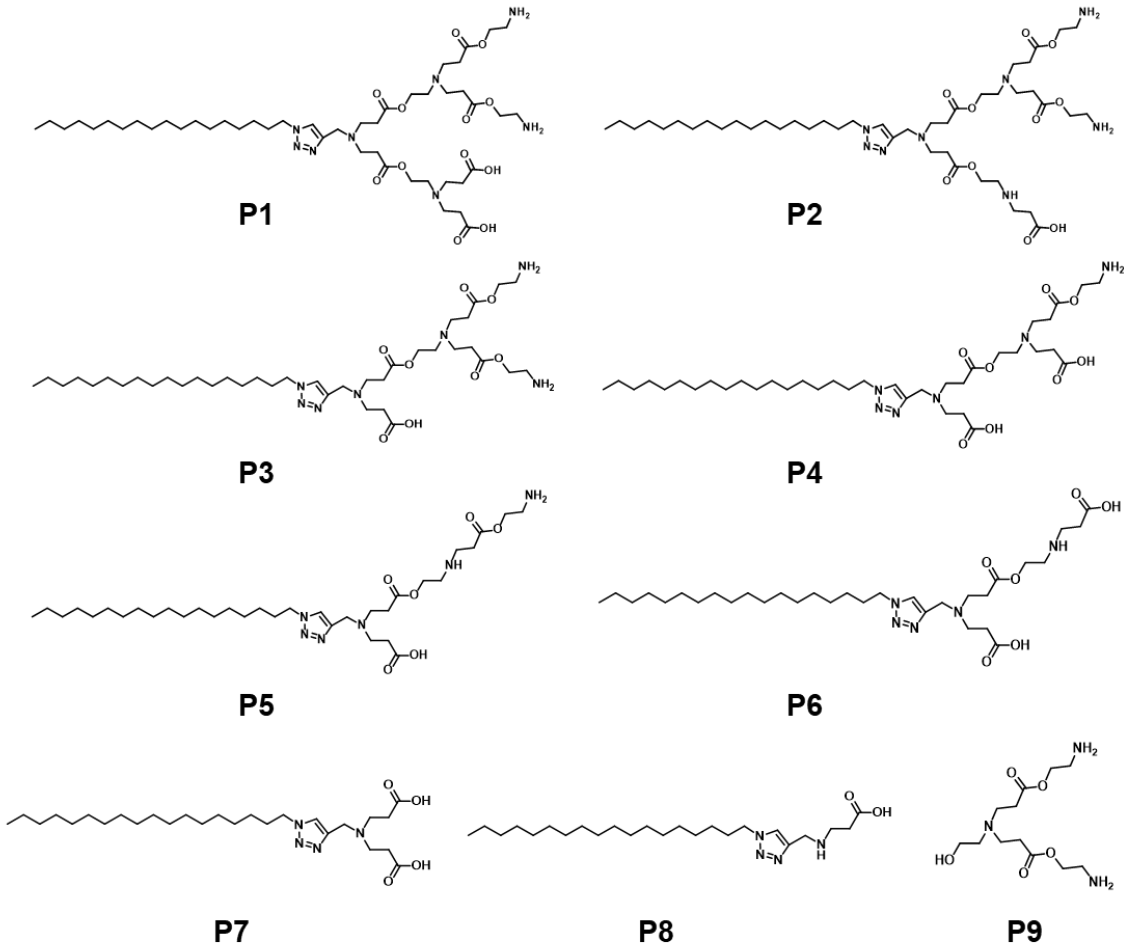


Fig. S3 Agarose gel retardation assay of siRNA stability against RNase in presence of dendrimer **I** at different time points. Naked siRNA was served as a control. (siRNA 200 ng per well, N/P ratio of 10)

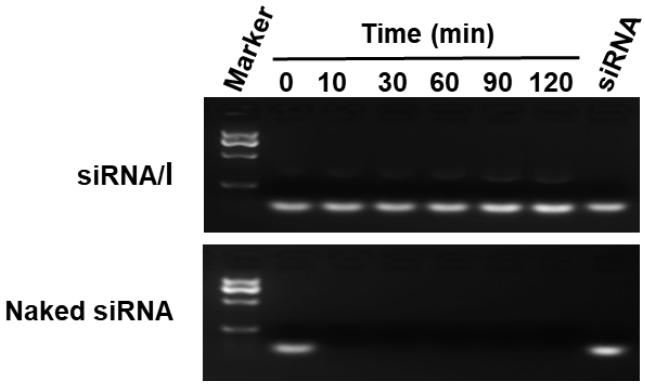


Fig. S4 (A) Flow cytometry analysis of the cellular uptake kinetics of siRNA/**l** complex (20 nM Cy5-labeled siRNA, N/P ratio of 10) on SKOV-3 cells after different incubation times (15, 30, 45 and 60 min). (mean ± SD, n = 3) (B) Confocal imaging of the cellular uptake of siRNA/**l** complex (20 nM Cy5-labeled siRNA, N/P ratio of 10) in SKOV-3 cells, evaluated using confocal microscopy. The red channel image shows the Cy5-labeled siRNA (red), and the blue channel image shows the nuclei of the SKOV-3 cells stained by Hoechst33342 (blue).

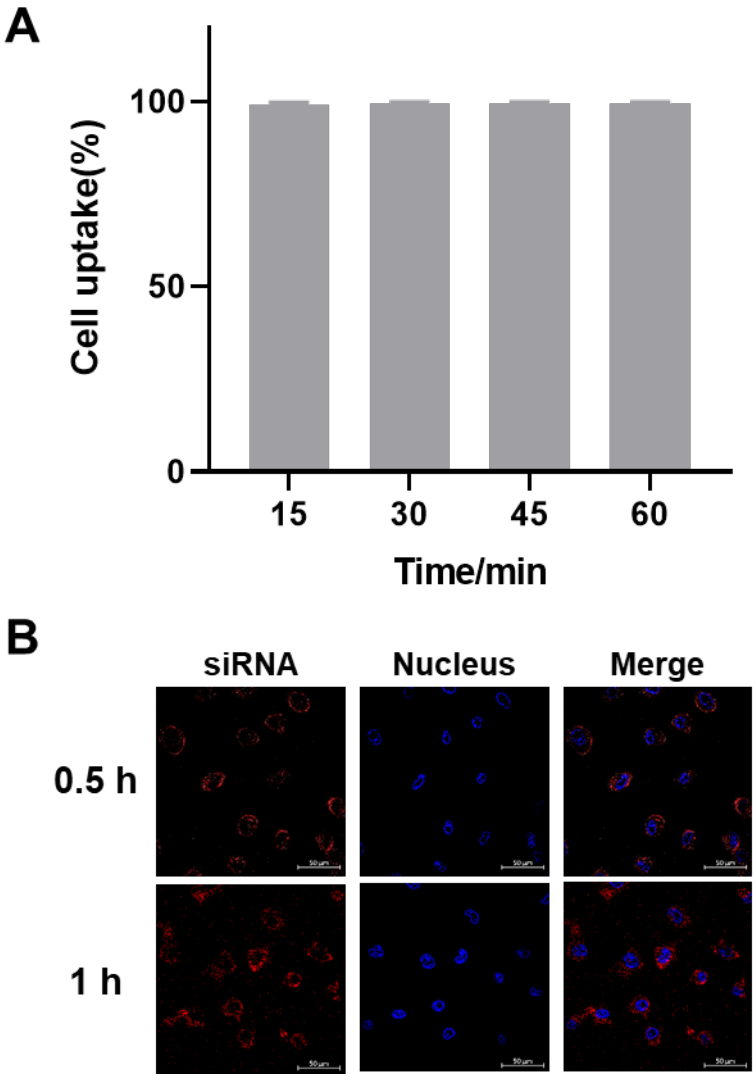


Fig. S5 Specific and potent silencing of AKT2 following **l**-mediated siRNA delivery in A549 cells and SKOV-3 cells by western blot evaluation (20 nM siAKT2, N/P ratio of 10). siAKT2: siRNA targeting AKT2; scramble: scramble siRNA.

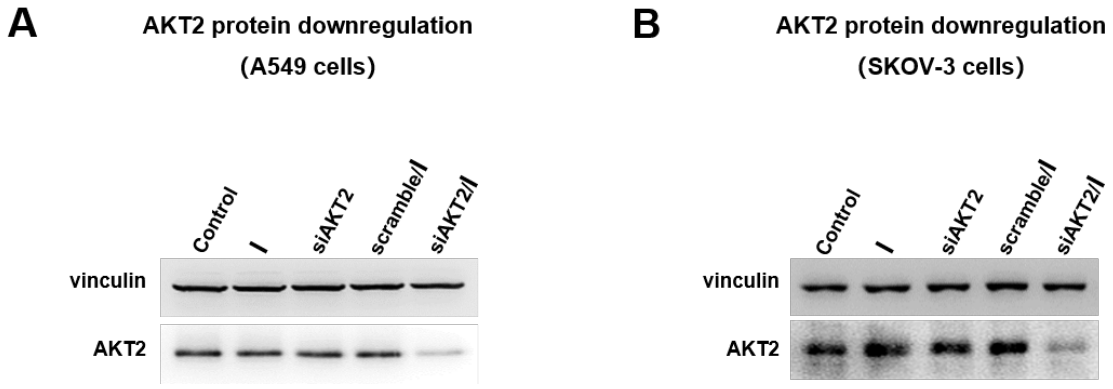


Fig. S6 Dose-dependent gene silencing of AKT2 in A549 cells upon treatment with the siAKT2/**l** complex using varying siAKT2 concentrations from 1.0 nM to 50 nM at N/P ratio of 10 by western blotting. siAKT2: siRNA targeting AKT2.

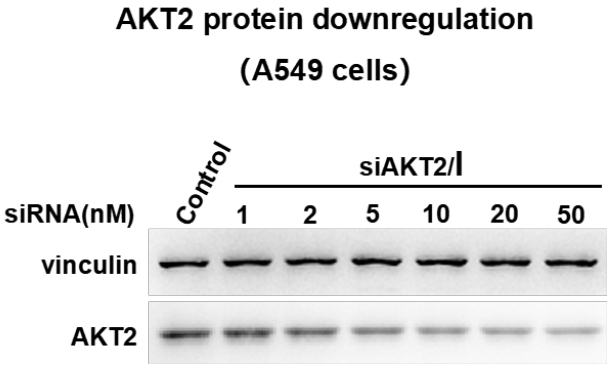


Fig. S7 AKT2 gene silencing in SKOV-3 cells following **I**- and **II**-mediated siRNA delivery, respectively. (20 nM siAKT2, N/P ratio of 10) siAKT2: siRNA targeting AKT2

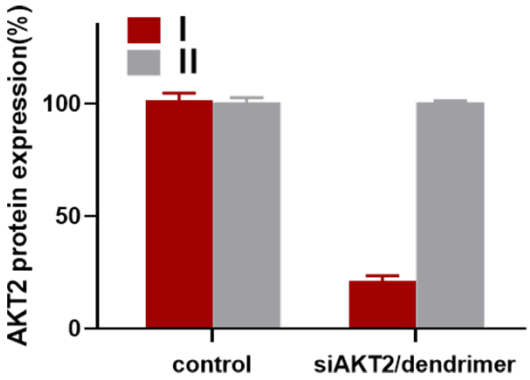


Fig. S8 Haemolysis assay of **I** or **II** at different concentrations using mouse red blood cells. Quantitative analysis of haemolysis was performed by recording UV absorption at 540 nm. (mean ± SD, n = 3)

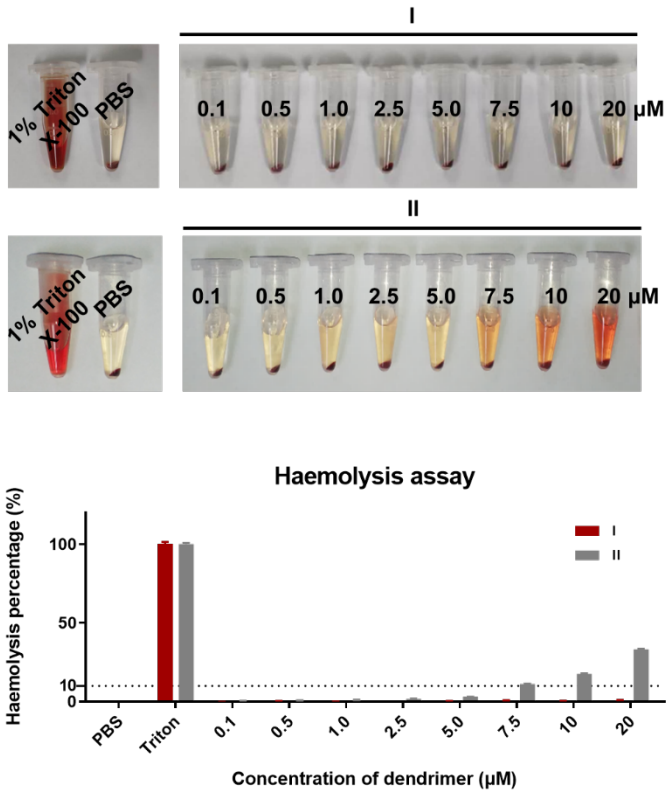


Fig. S9 siRNA release from the siRNA/dendrimer complexes was evaluated using heparin-coupled ethidium bromide (EB) fluorescence assays (mean ± SD, n = 3).

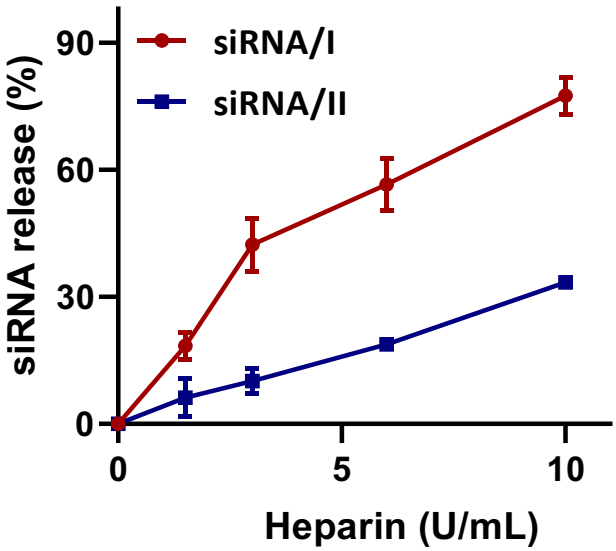


Fig. S10 (A) MTT assay of the metabolic toxicity and (B) LDH assay of the membrane damage toxicity of **I**-mediated siRNA delivery system. The control cells were treated with lysis buffer as 100% LDH release. (20 nM siRNA, N/P ratio of 10) (C) Hemolysis assay of siRNA/**I** complex using varying dendrimer **I** concentrations from 0.1 μM to 20 μM at N/P ratio of 10. Quantitative analysis of hemolysis was performed by recording UV absorption at 540 nm. (mean ± SD, n = 3)

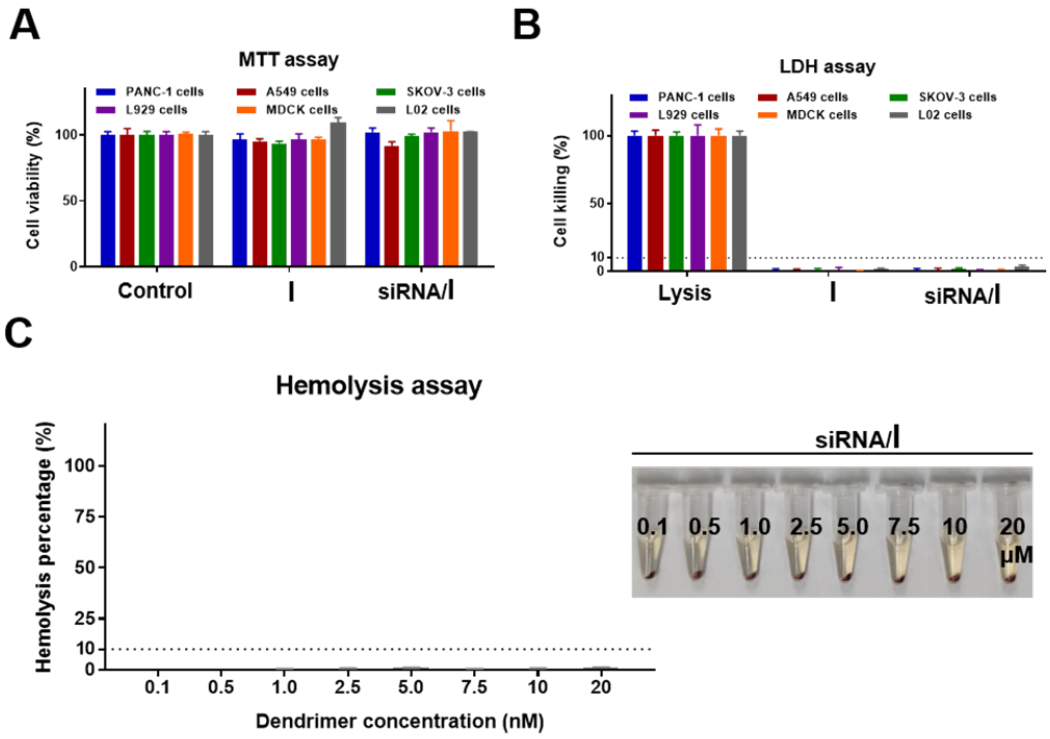


Fig. S11 In vivo toxicity evaluation of **l**-mediated siRNA delivery system. (A) Cytokine response in mice treated with Control PBS, **l**, and siRNA/**l** complex was determined by quantifying the serum levels of IL-1β, TNF-α and IL-6. Lipopolysaccharide (LPS) was used as the positive control. (mean ± SD, n = 3) (B) Major serum biochemistry parameters measured in mouse serum following the treatment of Control PBS, **l**, and siRNA/**l** complex. Unit for alanine transaminase (ALT), and aspartate transaminase (AST) is U/L, for triacylglycerol (TG) and cholesterol (CHO) is mmol/L, for total protein (TP) is g/L, for UREA is mg/dL, for creatinine (CREA) is μmol/L, respectively. (C) Histopathological analysis of major organs from mice treated with Control PBS, **l**, and siRNA/**l** complex. (1.0 mg/kg, N/P ratio of 5)

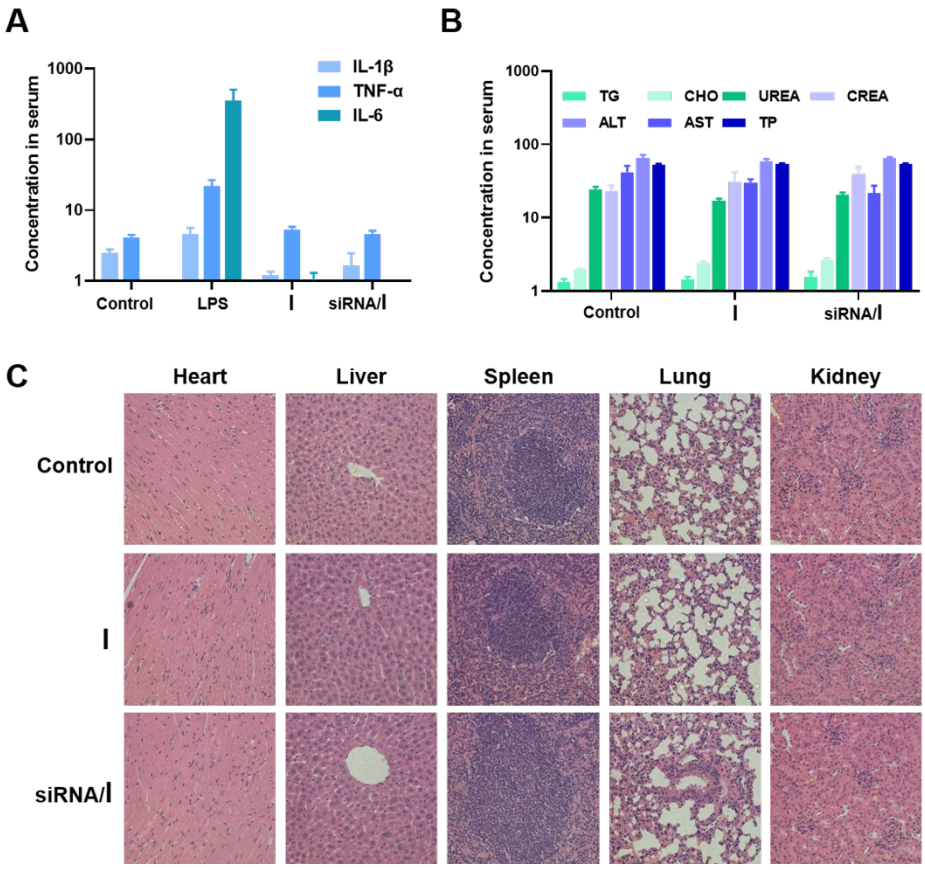
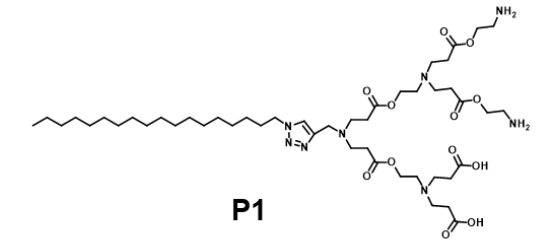
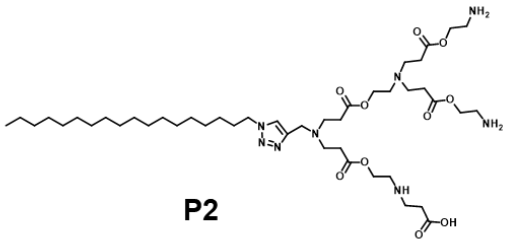


Table S1 Accurate mass measurements and proposed structures of degradation products upon treatment of the esterase (PLE).

Compound	Elemental composition	*m*/Zth	*m*/Zexp	Error (ppm)
P1	$C_{47}H_{87}N_8O_{12}^+$	955.6438	955.6440	+0.2
P2	$C_{44}H_{83}N_8O_{10}^+$	883.6227	883.6225	-0.2
P3	$C_{39}H_{74}N_7O_8^+$	768.5593	768.5587	-0.8
P4	$C_{37}H_{69}N_6O_8^+$	725.5171	725.5165	-0.8
P5	$C_{34}H_{65}N_6O_6^+$	653.4960	653.4946	-2.1
P6	$C_{32}H_{60}N_5O_6^+$	610.4538	610.4530	-1.3
P7	$C_{27}H_{51}N_4O_4^+$	495.3905	495.3904	-0.2
P8	$C_{24}H_{47}N_4O_2^+$	423.3694	423.3687	-1.6
P3	$C_{39}H_{75}N_7O_8^{2+}$	384.7833	384.7834	+0.3
P4	$C_{37}H_{70}N_6O_8^{2+}$	363.2622	363.2626	+1.1
P9	$C_{12}H_{26}N_3O_5^+$	292.1867	292.1873	+2.1



P1



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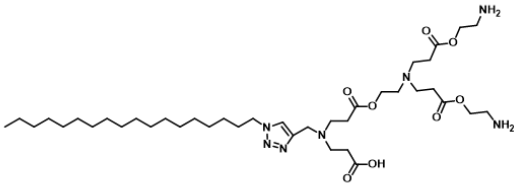

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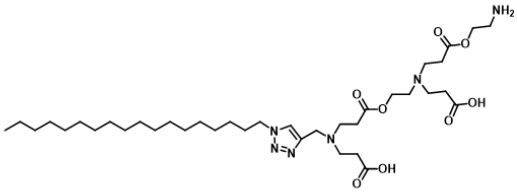

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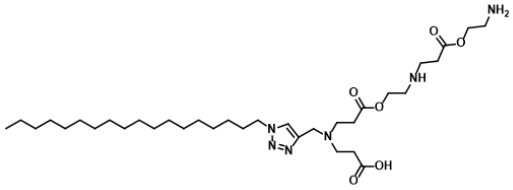

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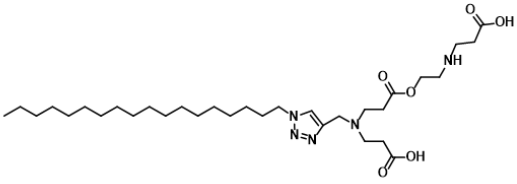

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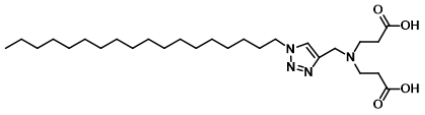

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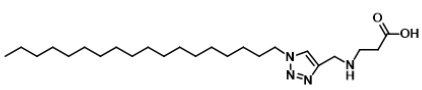

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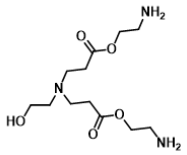


P9

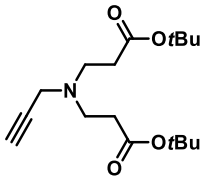
Synthesis and characterization of amphiphilic poly(aminoester) dendrimer

All the reagents and solvents were purchased from Aladdin Ltd. (Shanghai, China), Energy Chemical Ltd. (Shanghai, China) or Sigma-Aldrich (Shanghai, China). Tert-butyl acrylate was distilled before use. All the other reagents were used without any further purification from commercial sources. All the solvents were used after further distillation from commercial sources.

^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra recorded at 75 or 126 MHz on Bruker Avance 300 and 500 spectrometers (Bruker, Baden-Wuerttemberg, Germany), respectively. Coupling constants (*J*) are reported in Hertz, and chemical shifts are reported in parts per million (ppm).

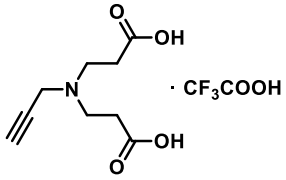
ESI-MS experiments were performed using a Waters Q-TOF MicroTM mass spectrometer (Waters Corporation, Framingham, MA, USA). High resolution MS (HRMS) experiments were performed using a Waters Xevo G2-XS Qtof mass spectrometer (Waters Corporation, Framingham, MA, USA) equipped with an electrospray ionization source operated in the positive mode. Infrared spectra were recorded with an ALPHA FT-IR spectrometer (Bruker, Baden-Wuerttemberg, Germany).

Poly(aminoester) dendrons (**1**, **2** and **3**) and the azido-bearing C18 alkyl chain (**7**) were synthesized according to the literature.[1-3]



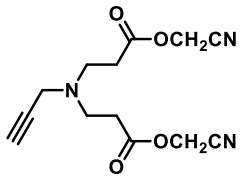
1

1: Propargylamine (220 mg, 3.99 mmol) was dissolved in anhydrous MeOH (5.4 mL) under nitrogen atmosphere in the dark, to which tert-butyl acrylate was added at 0°C. Then the mixture stirred at 30°C for 72 h. Solvents and reagents were removed under vacuum to give a yellowish crude oil product, which was further purified by silica gel column chromatography (PE/EA = 10/1) to afford **1** (1.05 g, 84%) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.41 (d, *J* = 2.4 Hz, 2H), 2.79 (t, *J* = 7.2 Hz, 4H), 2.37 (t, *J* = 7.2 Hz, 4H), 2.18 (t, *J* = 2.4 Hz, 1H), 1.44 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.71, 80.41, 78.39, 73.20, 49.21, 41.97, 34.41, 28.17. ESI-MS calcd for $C_{17}H_{29}NO_4$ $[M+H]^+$ 312.2, found 312.2.



2

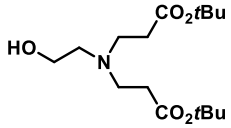
2: **1** (200 mg, 0.642 mmol) in anhydrous DCM (9.6 mL) at 0°C was treated with TFA (0.96 mL, 12.8 mmol) in the dark. The mixture was stirred at room temperature for 48 h. After solvent evaporation, the residue was washed with anhydrous diethyl ether (20 mL × 3), yielding **2** (177 mg, 88%) as white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 4.20 (d, *J* = 2.4 Hz, 2H), 3.54 (t, *J* = 6.6 Hz, 4H), 3.35 (t, *J* = 2.4 Hz, 1H), 2.87 (t, *J* = 6.6 Hz, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 174.06, 81.04, 72.85, 50.94, 43.73, 29.90.



3

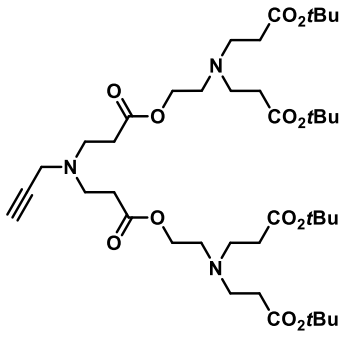
3: To a solution of compound **2** (270 mg, 0.862 mmol) in DMF (3.6 mL), NEt_3 (0.72

mL, 5.18 mmol) and then chloroacetonitrile (0.44 mL, 6.91 mmol) were added under ice bath conditions. The mixture was stirred at 30°C for 32 h. After solvent evaporation, the reaction mixture was extracted with EA, washed with distilled water solution (30 mL × 3), saturated NaCl solution (30 mL × 1), and then dried over Na_2SO_4. The crude product was further purified by silica gel column chromatography (PE/EA = 3/1), yielding **3** (204 mg, 86%) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.73 (s, 4H), 3.44 (d, *J* = 2.4 Hz, 2H), 2.88 (t, *J* = 6.6 Hz, 4H), 2.56 (t, *J* = 6.6 Hz, 4H), 2.23 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.70, 114.59, 77.61, 73.78, 48.73, 48.47, 41.77, 32.56.



4

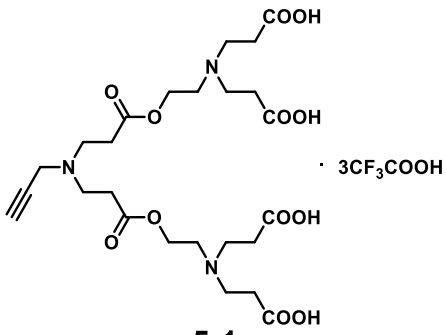
4: Ethanolamine (220 mg, 3.60 mmol) was dissolved in anhydrous MeOH (5.0 mL) under nitrogen atmosphere in the dark, to which tert-butyl acrylate was added at 0°C. Then the mixture stirred at 30°C for 48 h. Solvents and reagents were removed under vacuum to give a yellowish crude oil product, which was further purified by silica gel column chromatography (PE/EA = 5/1) to afford **2** (1.07 g, 94%) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.57 (t, *J* = 5.1 Hz, 2H), 3.00 (br, 1H), 2.76 (t, *J* = 6.9 Hz, 4H), 2.58 (t, *J* = 5.1 Hz, 2H), 2.37 (t, *J* = 6.9 Hz, 4H), 1.44 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.12, 80.72, 59.12, 55.79, 49.33, 33.80, 28.17. ESI-MS calcd for $C_{16}H_{31}NO_5$ $[M+H]^+$ 318.2, found 318.3.



5

5: Compound **4** (504 mg, 1.59 mmol) was dissolved in anhydrous MeCN (3.0 mL)

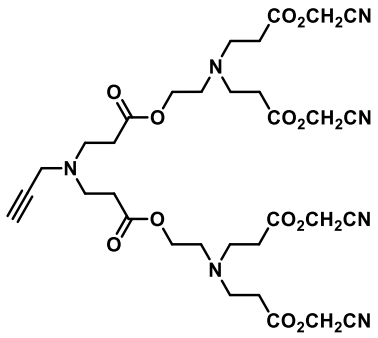
under nitrogen atmosphere in the dark, to which DBU (0.24 mL, 1.59 mmol) was added at 0°C. The MeCN (1.5 mL) of **3** was added to the mixture stirred at 30°C for 48 h. After solvent evaporation, the reaction mixture was extracted with ethyl acetate, washed with distilled water solution (30 mL × 3), saturated NaCl solution (30 mL × 1), and then dried over Na_2SO_4. The crude product was treated with benzoic anhydride (883 mg, 3.91 mmol) in the presence of DMAP (953 mg, 7.80 mmol) in DCM (10 mL) for 2 h at room temperature. The reaction mixture was extracted with DCM, washed with saturated $NaHCO_3$ solution (30 mL × 3), saturated NaCl solution (30 mL × 1), and then dried over Na_2SO_4. The crude product was further purified by silica gel column chromatography with PE/EA (2/1), yielding **5** (219 mg, 76%) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.11 (t, *J* = 6.3 Hz, 4H), 3.41 (d, *J* = 2.1 Hz, 2H), 2.90 – 2.73 (m, 12H), 2.70 (t, *J* = 6.0 Hz, 4H), 2.46 (t, *J* = 7.2 Hz, 4H), 2.35 (t, *J* = 7.2 Hz, 8H), 2.19 (t, *J* = 2.1 Hz, 1H), 1.44 (s, 36H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.25, 171.89, 80.48, 78.17, 73.48, 62.73, 52.17, 50.08, 49.02, 41.96, 34.11, 33.06, 28.21. ESI-MS calcd for $C_{41}H_{71}N_3O_{12}$ $[M+H]^+$ 798.5, found 798.6.



5-1

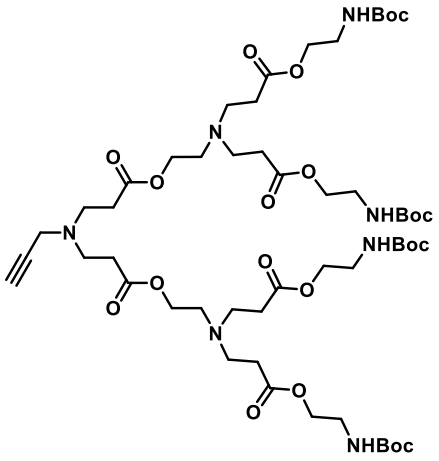
5-1: Compound **5** (170 mg, 0.213 mmol) in anhydrous DCM (6.4 mL) at 0°C was treated with TFA (0.64 mL, 8.52 mmol) in the dark. The mixture was stirred at room temperature for 48 h. After solvent evaporation, the residue was washed with anhydrous diethyl ether (20 mL × 3), yielding **5-1** (187 mg, 95%) as white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 4.55 (t, *J* = 4.8 Hz, 4H), 4.15 (d, *J* = 2.4 Hz, 2H), 3.68 – 3.45 (m, 16H), 3.28 (t, *J* = 2.7 Hz, 1H), 3.02 – 2.82 (m, 12H). ^{13}C NMR (75 MHz, CD_3OD) δ 174.56, 171.43, 81.36, 72.64, 60.01, 53.32, 51.40, 50.33, 43.91, 30.06,

28.99. ESI-MS calcd for $C_{25}H_{39}N_3O_{12}$ $[M+H]^+$ 574.2, found 574.3.



5-2

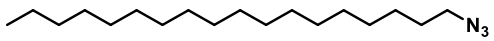
5-2: To a solution of compound **5-1** (180 mg, 0.197 mmol) in DMF (4.0 mL), NEt_3 (0.34 mL, 2.36 mmol) and then chloroacetonitrile (0.20 mL, 3.58 mmol) were added under ice bath conditions. The mixture was stirred at 30°C for 32 h. After solvent evaporation, the reaction mixture was extracted with EA, washed with distilled water solution (30 mL × 3), saturated NaCl solution (30 mL × 1), and then dried over Na_2SO_4. The crude product was further purified by silica gel column chromatography with PE/EA (1/2), yielding **5-2** (115 mg, 82%) as colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.93 (s, 8H), 4.01 (t, J = 5.9 Hz, 4H), 3.36 (d, J = 2.0 Hz, 2H), 3.09 (t, J = 1.9 Hz, 1H), 2.83 – 2.58 (m, 16H), 2.53 (t, J = 6.6 Hz, 8H), 2.41 (t, J = 7.0 Hz, 4H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.75, 171.09, 116.00, 78.83, 75.69, 61.95, 51.29, 48.89, 48.84, 48.51, 41.36, 32.42, 31.71. ESI-MS calcd for $C_{33}H_{43}N_7O_{12}$ $[M+H]^+$ 730.3, found 730.4.



6

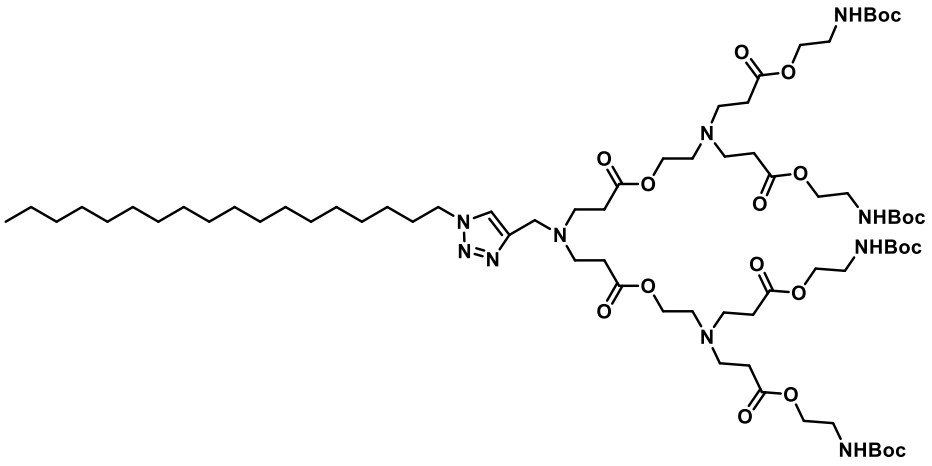
6: Compound **5-2** (245 mg, 0.336 mmol) and N-Boc-ethanolamine (303 mg, 1.88

mmol) were dissolved in anhydrous MeCN (9.0 mL) under nitrogen atmosphere, to which DBU (0.28 mL, 1.88 mmol) was added dropwise at room temperature. The solution was stirred at 30°C for 10 h. After solvent evaporation, the reaction mixture was extracted with DCM, washed with distilled water solution (30 mL × 3), saturated NaCl solution (30 mL × 1), and then dried over Na_2SO_4. The crude product was further purified by silica gel column chromatography with DCM/EtOH (30/1), yielding **6** (258 mg, 67%) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.11 (s, 4H), 4.20 – 4.06 (m, 12H), 3.49 – 3.27 (m, 10H), 2.83 (t, *J* = 6.9 Hz, 12H), 2.72 (t, *J* = 6.2 Hz, 4H), 2.47 (t, *J* = 6.9 Hz, 12H), 2.21 (t, *J* = 2.3 Hz, 1H), 1.44 (s, 36H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.38, 172.26, 155.96, 79.59, 78.10, 77.36, 73.60, 63.82, 62.31, 52.15, 49.91, 49.00, 41.89, 39.71, 33.02, 32.96, 28.50. ESI-MS calcd for $C_{53}H_{91}N_7O_{20}$ $[M+H]^+$1146.6, found 1146.8.



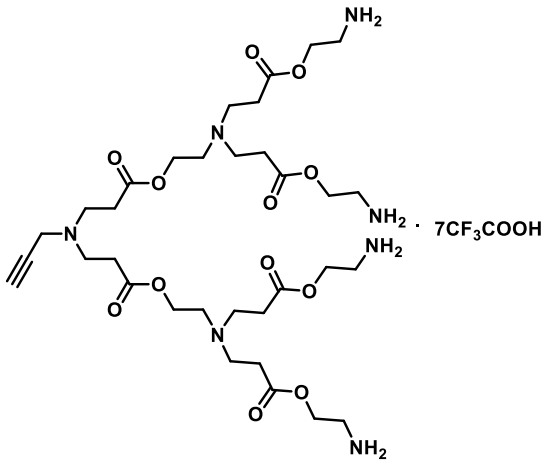
7

7: NaN_3 (98.0 mg, 1.50 mmol) and 1-Bromooctadecane (200 mg, 0.600 mmol) were added into anhydrous DMF (2.5 mL) under nitrogen atmosphere. The solution was stirred at 65°C for 12 h. After solvent evaporation, the reaction mixture was extracted with EA, washed with distilled water solution (30 mL × 3), saturated NaCl solution (30 mL × 1), and then dried over Na_2SO_4. The crude product was further purified by silica gel column chromatography with PE, yielding **7** (159 mg, 90%) as colorless oil.^{1}H NMR (300 MHz, $CDCl_3$) δ 3.25 (t, *J* = 7.0 Hz, 2H), 1.68 – 1.53 (m, 2H), 1.47 – 1.13 (m, 30H), 0.88 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 51.64, 32.08, 29.85, 29.82, 29.78, 29.70, 29.64, 29.52, 29.31, 28.99, 26.87, 22.85, 14.27. IR (cm^{-1}): *υ* 2092.38 ($-N_3$).



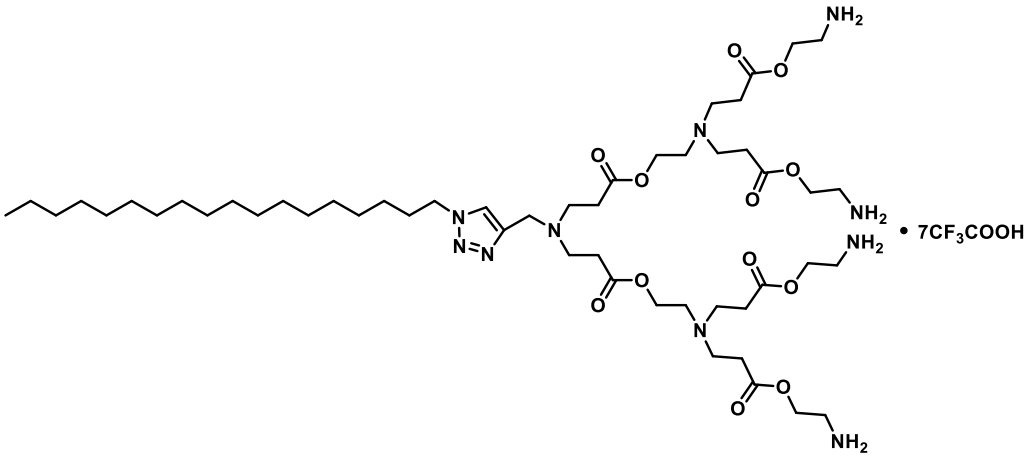
8

8: **7** (120 mg, 0.105 mmol), **6** (33.0 mg, 0.110 mmol) and CuI (10.0 mg, 52.0 μmol) were dissolved in anhydrous DMF (7.0 mL) under argon atmosphere, to which DBU (0.12 mL, 0.825 mmol) was added at room temperature. The solution was stirred at 50°C in the dark for 3 h. After solvent evaporation, the reaction mixture was extracted with DCM, washed with saturated NH_4Cl solution (30 mL × 3), brine (30 mL × 1), and then dried over Na_2SO_4. The crude product was further purified by silica gel column chromatography with DCM/EtOH (30/1), yielding **8** (122 mg, 81%) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 (s, 1H), 5.16 (s, 4H), 4.32 (t, *J* = 7.3 Hz, 2H), 4.20 – 4.05 (m, 12H), 3.80 (s, 2H), 3.38 (q, *J* = 5.1 Hz, 8H), 2.91 – 2.64 (m, 16H), 2.57 – 2.41 (m, 12H), 1.96 – 1.82 (m, 2H), 1.44 (s, 36H), 1.36 – 1.19 (m, 30H), 0.87 (t, *J* = 6.3 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.45, 172.37, 155.96, 144.16, 122.53, 79.53, 63.80, 62.28, 52.09, 50.41, 49.88, 48.76, 48.34, 39.66, 32.91, 32.59, 32.00, 30.45, 29.78, 29.74, 29.71, 29.64, 29.52, 29.44, 29.13, 28.48, 26.62, 22.78, 14.23. ESI-MS calcd for $C_{71}H_{128}N_{10}O_{20}$ $[M+H]^+$ 1441.9, found 1442.2.



9

9: To a solution of **6** (26.0 mg, 23.0 μmol) in DCM (1.0 mL), TFA (0.50 mL, 6.80 mmol) was added under ice bath conditions. The mixture was stirred at 30°C for 6 h. After solvent evaporation, the residue was washed with anhydrous diethyl ether (10 mL × 3), yielding **9** (33.0 mg, 95%) as white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 4.54 (t, *J* = 4.8 Hz, 4H), 4.40 (t, *J* = 5.1 Hz, 8H), 4.10 (d, *J* = 2.2 Hz, 2H), 3.61 (t, *J* = 6.6 Hz, 12H), 3.50 (t, *J* = 6.8 Hz, 4H), 3.30 – 3.24 (m, 9H), 3.03 (t, *J* = 6.8 Hz, 8H), 2.95 (t, *J* = 6.8 Hz, 4H).



I

I: To a solution of **8** (27.0 mg, 19.0 μmol) in DCM (0.74 mL), TFA (0.26 mL, 3.37 mmol) was added under ice bath conditions. The mixture was stirred at 30°C for 6 h. After solvent evaporation, the residue was washed with anhydrous diethyl ether (10 mL × 3), yielding **I** (33.0 mg, 96%) as white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 8.21 (s, 1H), 4.57 – 4.31 (m, 16H), 3.56 – 3.34 (m, 16H), 3.27 (t, *J* = 5.4, 8H), 3.08 – 2.87

(m, 12H), 1.99 – 1.85 (m, 2H), 1.44 – 1.22 (m, 30H), 0.90 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, MeOD) δ 171.96, 171.46, 137.46, 128.10, 62.53, 60.60, 53.55, 51.63, 50.75, 49.93, 39.75, 33.00, 31.19, 30.71, 30.68, 30.62, 30.49, 30.39, 30.06, 29.92, 29.72, 27.46, 23.66, 14.38. HRMS calcd for $C_{51}H_{96}N_{10}O_{12}$ $[M+H]^+$ 1041.7282, found 1041.7246 (-3.5 ppm).

Mass Spectrometry

ESI-MC measurements: Prior to MS analysis, the compounds were dissolved in chloroform or dimethyl sulfoxide, the sample solutions were introduced in the ionization source using HPLC (flow rate: 0.2 mL min^{-1}). The capillary voltage was set at +2300 V and the cone voltage at +25 V. Instrument control, data acquisition and data processing of all experiments were achieved using MassLynx 4.0 programs provided by Waters.

HRMS measurements: Prior to HRMS analysis, the samples were dissolved in methanol with 1% formic acid (v/v) before injection. The capillary voltage was set at +2.0 KV and the cone voltage at +40 V. An internal standard is used. Nitrogen was used as desolvation gas at 600L/h as well as the collision gas. Instrument control, data acquisition and data processing of all experiments were achieved using Masslynx V4.2 software provided by Waters.

Materials

The human AKT2 siRNA (sense: 5'-GCUCCUUCAUUGGGUACAAdTdT-3'; antisense: 5'-UAAUGUGCCCGUCCUUGUCdTdT-3'), scramble siRNA (sense: 5'-CUUACGCUGAGUACUUCGAdTdT-3'; antisense: 5'-UCGAAGUACUCAGCGUAAG dTdT-3') and Cy-5 labeled scramble siRNA were purchased from Guangzhou Ruibo (Guangzhou, China). Hoechst33342, Lyso-Tracker Red, RNase, Heparin and TritonX-100 solution were purchased from Beyotime Biotechnology (Shanghai, China). Anti-

GAPDH mouse polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc., (Beijing, China). Anti-human AKT2 rabbit polyclonal antibody were purchased from Cell signaling Technology (Massachusetts, USA). Anti-rabbit monoclonal secondary antibodies and anti-mouse monoclonal secondary antibodies were purchased from Invitrogen (Massachusetts, USA). Dioleoylphosphatidylethanolamine (DOPE), bafilomycin A1, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), porcine liver esterase (PLE), anti-human vinculin rabbit polyclonal antibody and lipopolysaccharide (LPS) were supplied by Sigma-Aldrich (Shanghai, China). FBS were purchased from Gemini Bio-Products (California, USA). Commercial Lactate dehydrogenase (LDH) assay kit was purchased from Dojindo Laboratories (Shanghai, China). GoodView nucleic acid dyes were purchased from SBS Genetech Co., Ltd. (Beijing, China). Phosphate buffered saline (PBS) was purchased from Servicebio (Wuhan, China). All other reagents and solvents of analytical grade were used without further purification from commercial sources.

Enzymatic hydrolysis assay

Degradation of **I** upon treatment of the esterase (PLE) was analyzed by HRMS at different timed lapses (0 and 24 h). Dendrimer **I** (5.0 mg) was dissolved in 700 μL of deuterated phosphate buffer (PB, pH = 7.4). The solution was then incubated with 4.0 mg PLE in a shaker at 37°C for different time lapses (0 and 24 h). At different time lapses (0 and 24 h), 1.40 mL of acetonitrile was added to the above mixture to stop the enzymatic hydrolysis. Then the sample was subject to centrifugation at 12000 rpm for 5 min, and the supernatant was taken for HRMS measurement.

Dynamic light scattering (DLS)

The siRNA solution was mixed with certain amount of dendrimer **I** solution at N/P ratio of 10. The final concentration of the siRNA was 1.0 μM. The sizes and zeta

potentials of the siRNA/**I** complex solutions were measured using a NanoBrookOmni (Brookhaven, State of New York, USA) equipped with a standard 633 nm laser at 25 °C. The experiments were performed in triplicates.

Gel retardation analysis

The dendrimer **I** was diluted to an appropriate concentration. The siRNA was dissolved in H_2O. Both solutions were mixed with at different N/P ratios from 0.2 to 10 and incubated at 37°C for 30 min. The final concentration of siRNA in each sample was 200 ng/well. The siRNA/**I** complex were analyzed by electrophoretic mobility-shift assays in 1% agarose gel in standard MOPS buffer for 15 min. The siRNA bands were stained by GoodView nucleic acid dyes and detected by Automatic chemiluminescence imaging system (Tanon, Shanghai, China).

Stability assessment of siRNA in presence of dendrimer

An aliquot of 1.4 μg of siRNA and the indicated amounts of dendrimer **I** solutions at N/P ratio of 10 were kept at 37 °C for 30 min. Afterwards, the siRNA/**I** complex were incubated in the presence of RNase A (1.0 μg/mL) at 37 °C for different times (0, 10, 30, 60, 90, 120 min). Aliquots (8.0 μL) of the corresponding solution were withdrawn and added to 0.50 μL of 1% SDS solution on ice. The mixtures were electrophoresed through a 1% agarose gel in standard TAE buffer. The siRNA bands were stained using nucleic acid dyes and then detected using Automatic chemiluminescence imaging system (5200Muti) (Tanon). Naked siRNA was served as a control.

RNA dissociation assay

In black 96-well plate, 1.8 μg ethidium bromide (EB) and 3.6 μg siRNA were mixed in PBS buffer at pH 7.4 and incubated for 15 min at 25 °C. The appropriate amount of

dendrimer **Ⅰ** or **Ⅱ** diluted in PBS at N/P ratio of 10 was incubated 10 min at 25 °C. Then the above dendrimer **Ⅰ** or **Ⅱ** solution was added to EB-siRNA solution and further incubated for 30 min at 25 °C. Then 40.0 μL heparin solution with different concentration (0, 1.5, 3, 6, 10 U/mL) diluted in PBS was added to the siRNA/ dendrimer complexes to achieve a total volume of 100 μL with the final concentration of EB at 1.0 μg/well, siRNA at 20 μM per base pair. The mixture was further incubated for 30 min at 25 °C. The fluorescence emission was recorded at the emission wavelength of 590 nm using Cytation5 Microplate Reader (BioTek, Vermont, USA). Excitation wavelength is 360 nm. The fluorescence values were normalized to wells containing only EB-siRNA solution. All samples were run in triplicate.

Cell culture

Human pancreatic cancer PANC-1 cells, human ovarian cancer SKOV-3 cells, human normal liver L02 cells, mouse fibroblast L929 cells, madin-darby canine kidney MDCK cells, and mouse fibroblast L929 cells were purchased from Tongpai Biotechnology Co., Ltd. (Shanghai, China). Human non-small cell lung cancer A549 cells were provided by Prof. Can Zhang at China Pharmaceutical University.

PANC-1 and L929 cells were cultured in RPMI-1640 (Hyclone, Utah, USA) with 10% FBS. A549 and L02 cells were cultured in DMEM (Hyclone) with 10% FBS. SKOV-3 cells were cultured in McCOY'S 5A (Hyclone) with 10% FBS. MDCK cells were maintained in MEM (Hyclone) with 10% FBS. Cells were maintained at 37°C with 5% CO_2 humidified atmosphere.

Flow cytometry

SKOV-3 cells were seeded at 5.0 × 10^4 cell/well in a 24-well plate one day before transfection. The cells were incubated with Cy5 labeled siRNA/**Ⅰ** complex (20 nM Cy5-siRNA, N/P ratio 10) for 10, 20, 30 and 60 minutes. The cells were then washed three

times with cold PBS and then analyzed by flow cytometry (Attune NxT, Thermo Fisher Scientific). Each assay was performed in triplicate.

Confocal microscopy

SKOV-3 cells were seeded in 2.5 dishes (8.0×10^4 cells/dish) one day before transfection. The cells were incubated with Cy5 labeled siRNA/**I** complex (20 nM Cy5-siRNA, N/P ratio 10) for 0.5, 1 and 2 h at 37°C. The cells were washed three times with cold PBS, and then PBS mixed with Hoechst33342 (10 μg/mL) and Lyso-Tracker Red (0.10 μM) was added, and stained for 10 min at 37°C. A Zeiss LSM880 Meta laser scanning confocal microscope (Carl Zeiss, Jena, Germany) was used for visualization. Images were acquired using ZEN2.3 pro software (Carl Zeiss GmbH).

In vitro transfection

PANC-1, A549 and SKOV-3 cells were seeded in 6-well plates (8.0×10^4 cells/well) and cultured in 2.0 mL of fresh medium containing 10% FBS one day before transfection. The siRNA/**I** complex solution was prepared at N/P ratio of 10 before transfection. The desired amount of siRNA and dendrimer **I** was diluted in 0.10 mL of Opti-MEM transfection medium. The solutions were mixed by pipette and then left for 10 min at room temperature. The dendrimer **I** was added to the siRNA solution, homogenized for 10 s and then left 30 min at room temperature. Then 0.80 mL of Opti-MEM medium was added in complex solution and the final volume brought to 1.0 mL. Before addition of the transfection complex, the complete medium with serum was removed and cells were washed by PBS once. Then, 1.0 mL of the complex solution was added and incubated at 37°C. After 8 h of incubation, the transfection mixture was replaced with the complete medium containing 10% FBS and maintained under normal growth conditions for further incubation of 72 h for Western blot assay.

Effect of DOPE: The transfection experiments involving DOPE were performed in

PANC-1 cells as described above, except the desired amount of dendrimer **I** was pre-mixed with DOPE at mole ratio of 0.25 (dendrimer/DOPE) and equilibrated at room temperature for 10 min before diluting in 0.1 mL of Opti-MEM transfection medium for the complexation with AKT2 siRNA.

Effect of bafilomycin A1: The transfection experiments involving bafilomycin A1 were performed as described above, except that PANC-1 cells were pre-incubated with 200 nM bafilomycin A1 for 1 h at 37^{o}C.

Western blot analysis

Samples containing equal amounts of proteins (15 μg) from cultured cells were separated by SDS-PAGE gradient gel, and transferred to the PVDF membrane after electrophoresis. Then PVDF membranes were blocked in 5% skimmed milk and incubated with anti-human vinculin rabbit polyclonal antibody or anti-GAPDH mouse polyclonal antibody or anti-human AKT2 rabbit polyclonal antibody at 4°C overnight. Then, after washing, the membranes were incubated with anti-rabbit or anti-mouse monoclonal secondary antibodies for 2 h at 25°C. Specific proteins were detected using an enhanced-chemiluminescence Western blotting analysis system (Tanon).

MTT assay

Cancer cell lines (PANC-1, A549 and SKOV-3 cells) and normal cell lines (MDCK, L02 and L929 cells) were respectively seeded in 96-well plates (8.0×10^3 cells/well) and allowed to attach overnight. The cells were then treated with dendrimer **I** and siRNA/**I** complex. After 8 h of treatment, the transfection mixture was replaced with the complete medium containing 10% FBS, and maintained under normal growth condition for further incubation of 48 h for cell metabolic toxicity assessment. MTT solution (0.50 mg/mL) was add to each well and incubated for another 4 h. And then the suspension liquid was removed and cells were resuspended in DMSO. The optical

density (OD) of these DMSO solutions was read at 570 nm using Cytation5 Microplate Reader (BioTek, Vermont, USA). The difference of OD values between treated and non-treated cells reflects the viability of cells after treatments and thus stands for the metabolite toxicity. Each assay was performed in triplicate.

LDH assay

Cancer cell lines (PANC-1, A549 and SKOV-3 cells) and normal cell lines (MDCK, L02 and L929 cells) were respectively seeded in 96-well plates (8.0×10^3 cells/well) and allowed to attach overnight. The cells were then treated with dendrimer **Ⅰ** and siRNA/**Ⅰ** complex for 8 h. Afterwards, cell membrane damage toxicity was determined using commercial LDH assay kit. The LDH reaction mixture was freshly prepared according to the manufacturer's protocol, 100 μL added to each well of a 96-well plate containing 100 μL of blank, control or treated cells. The cells were incubated for 30 min at 25°C followed by adding 50 μL of stop solution. The optical density (OD) of these solutions were measured at 490 nm via Cytation5 Microplate Reader (BioTek). Control was performed with lysis buffer and medium, and set as 100% and 0% LDH release, respectively. Each assay was performed in triplicate.

LDH% = [(the absorbance of sample – the absorbance of negative control) / (the absorbance of positive control – the absorbance of negative control)] ×100%.

Haemolysis experiment

Red blood cells (RBCs) were isolated from 1.0 mL fresh blood collected from healthy Kunming mice by centrifuged at 5×10^3 rpm for 4 min. The RBCs were washed several times with PBS buffer until no color was seen in the supernatant. RBCs were then suspended in 15 mL PBS, and 0.50 mL of such RBC suspension was added to 0.50 mL of suspension containing different concentrations of **Ⅰ** or **Ⅱ** assemblies at 0.2, 1.0, 2.0, 5.0, 15, 9.0, 20 and 40 μM in PBS buffer to offer the final concentration of 0.1, 0.5,

1.0, 2.5, 7.5, 10 and 20 μM, respectively. A 0.50 mL RBC suspension incubated with 0.50 mL PBS or 0.50 mL 1.0% TritonX-100 solution was used as negative and positive controls, respectively. The samples were mixed gently, left at room temperature for 2 h, and then centrifuged at 1.0×10^4 rpm for 5 min. A total of 100 μL of supernatant was transferred to a 96-well plate and the absorbance of hemoglobin at 540 nm was measured via Cytation5 Microplate Reader (BioTek). The percentage of haemolysis was calculated as follows:

Haemolysis% = [(the absorbance of sample – the absorbance of negative control) / (the absorbance of positive control – the absorbance of negative control)] ×100%.

Safety assessment in vivo

Institutional guidelines for the proper and human use of animals in research were followed. 5-week-old male ICR mice were purchased from Comparative Medicine Centre of Yangzhou University, China. Mice were randomized into five groups (3 mice per group), and were then intravenously treated with either, PBS buffer, dendrimer **I**, and scramble siRNA/**I** complex (1.0 mg/kg, N/P ratio of 5). After 24 hours, mice were sacrificed. The serum specimens were collected to measure the inflammatory factors and biochemical parameters. Tissue specimens of different organs (hearts, lungs, livers, kidneys, and spleens) were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 4 μm thickness were stained with haematoxylin and eosin (H&E) staining.

Statistical tests

All data are presented as mean ± SD unless otherwise indicated. Statistical analysis was performed by one-way ANOVA or two-way ANOVA with Tukey's post-hoc test (Graphpad Prism 8.01). $p \leq 0.05$ was considered significant (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).

References

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