PNAS

## Supporting Information for

# Cargo-selective and adaptive delivery of nucleic acid therapeutics by bola-amphiphilic dendrimers

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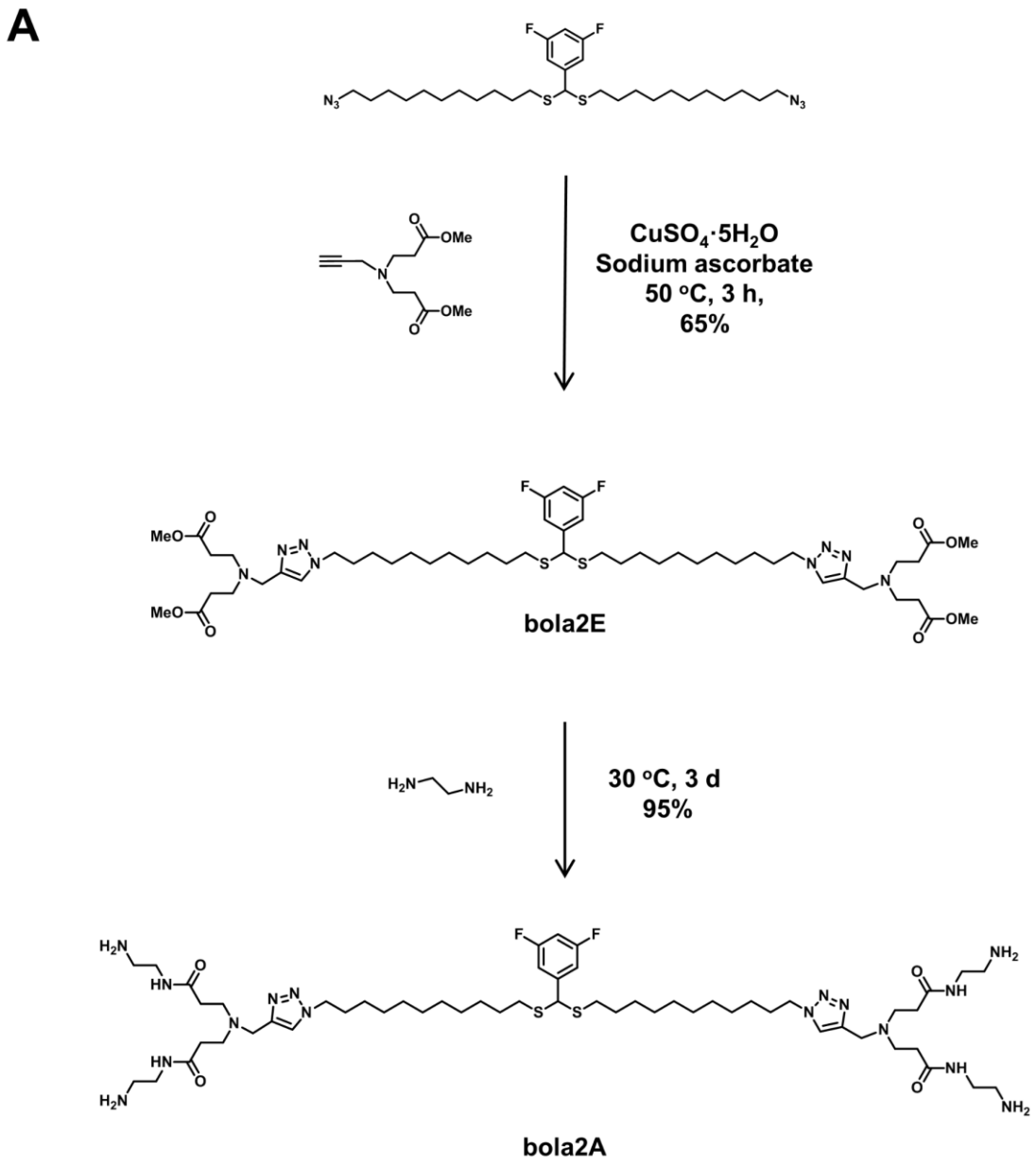
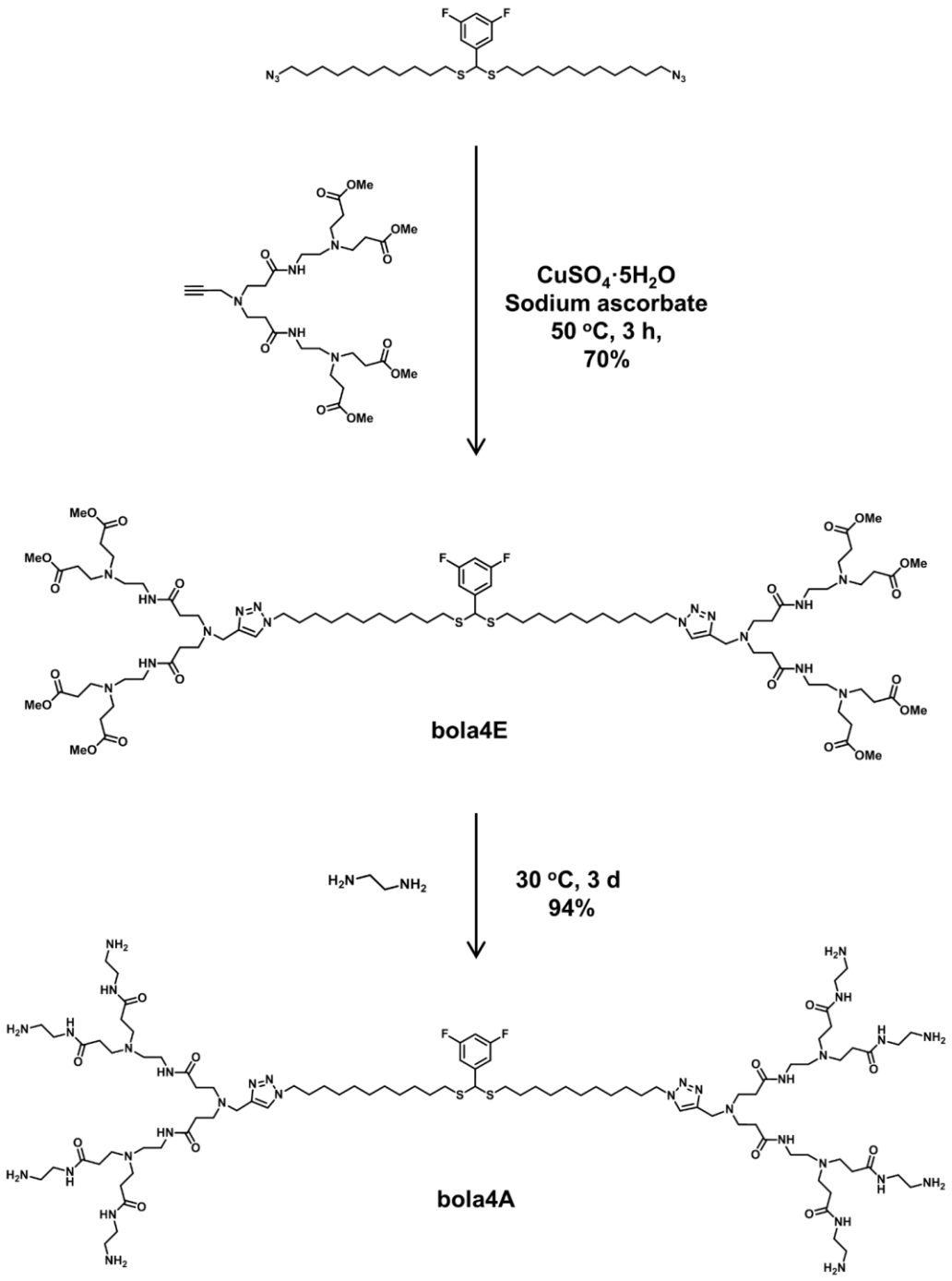
**Fig. S1.** Synthesis of **bola2A** (A), **bola4A** (B) and **bola8A** (C).



**B**


CuSO4·5H2O
Sodium ascorbate
50 °C, 3 h,
70%
bola4E
30 °C, 3 d
94%
bola4A

**C**

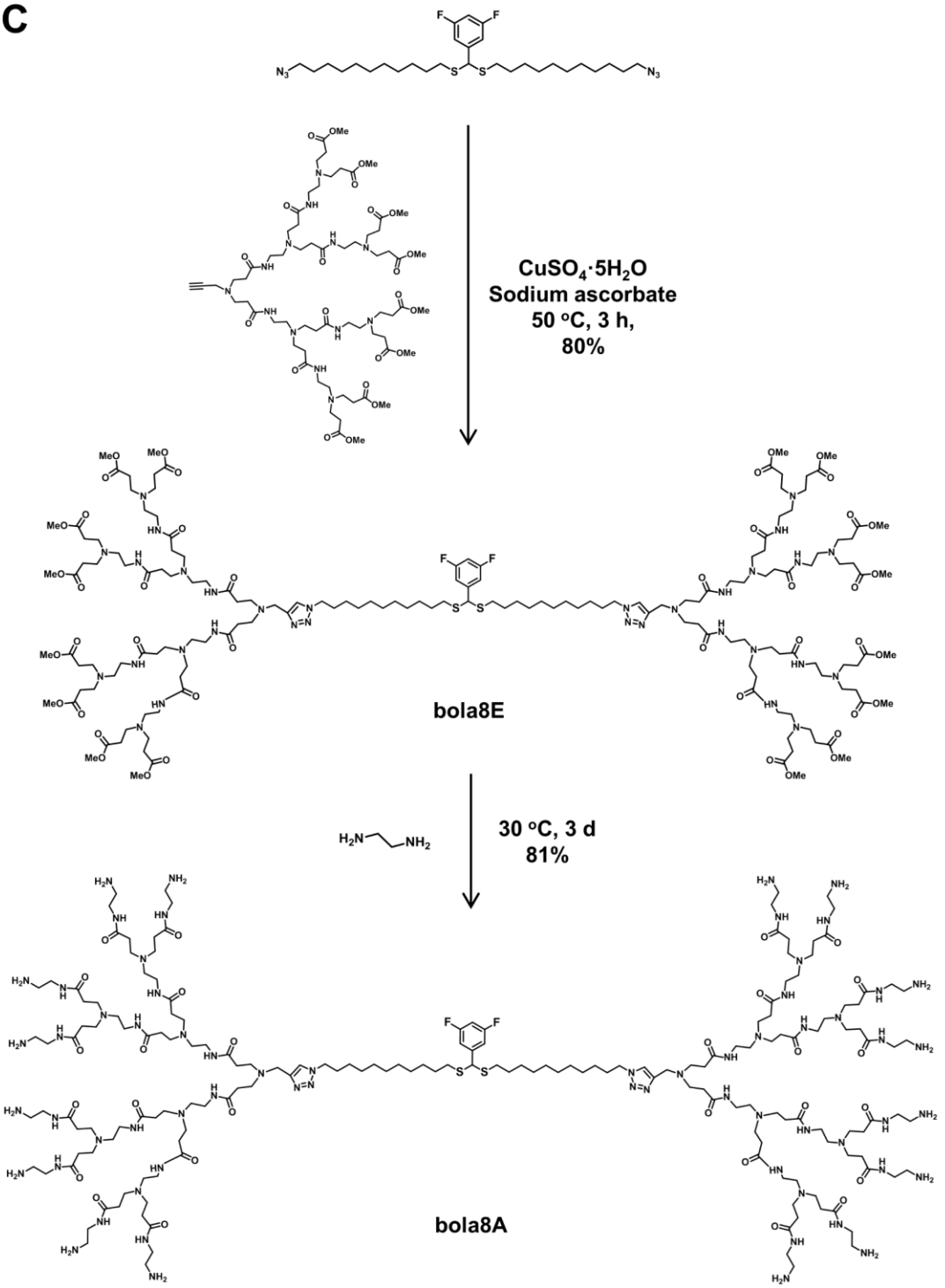
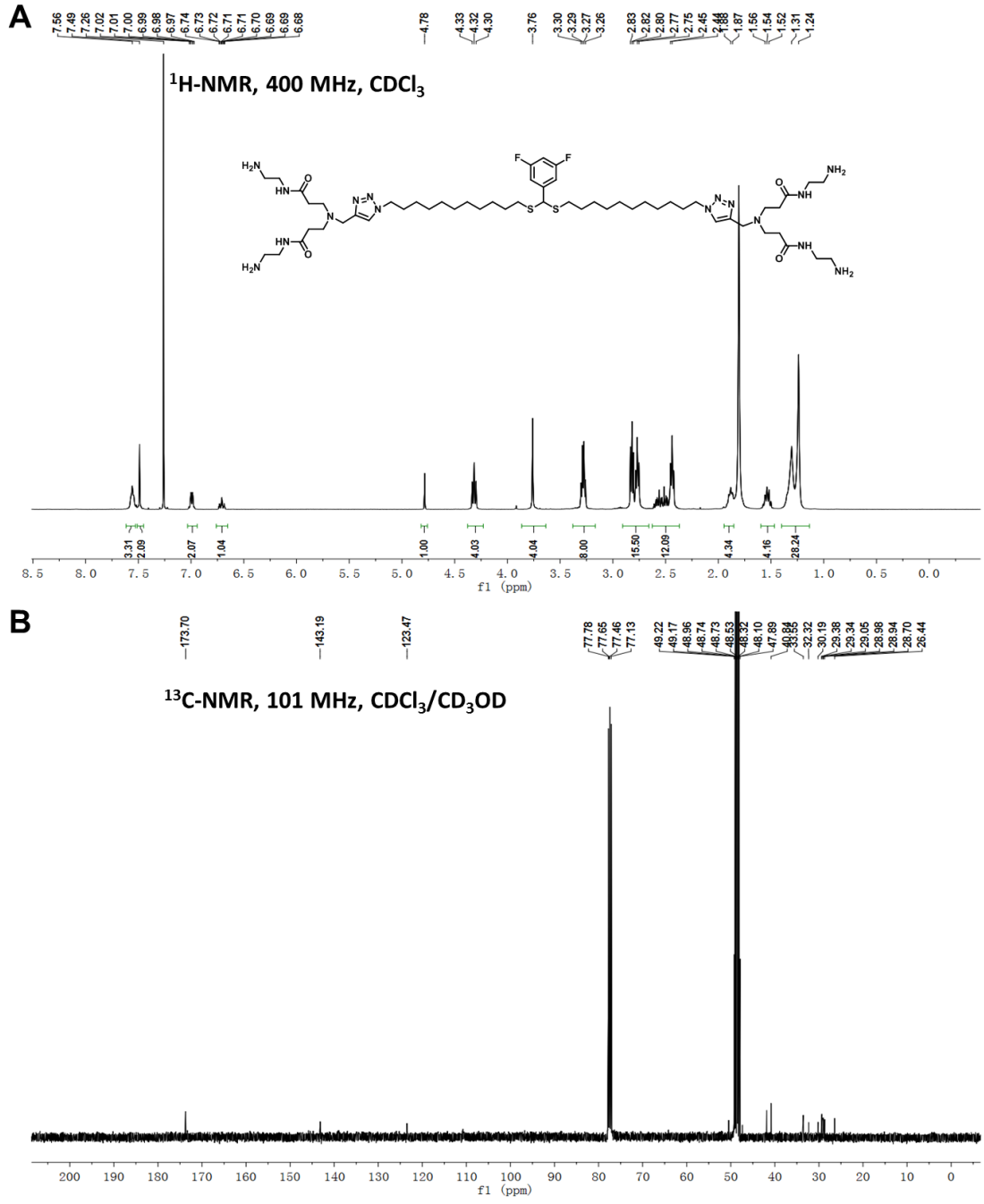
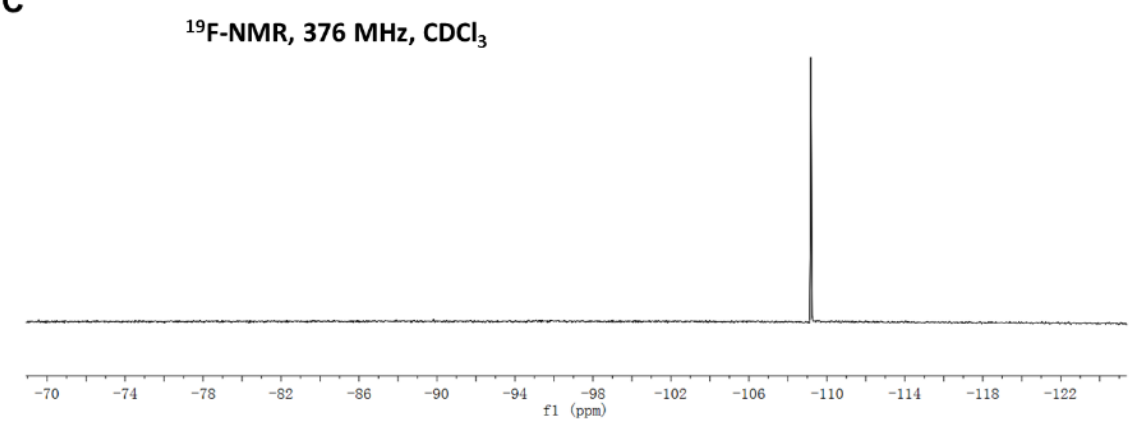

CuSO4·5H2O
Sodium ascorbate
50 °C, 3 h,
80%
bola8E
30 °C, 3 d
81%
bola8A

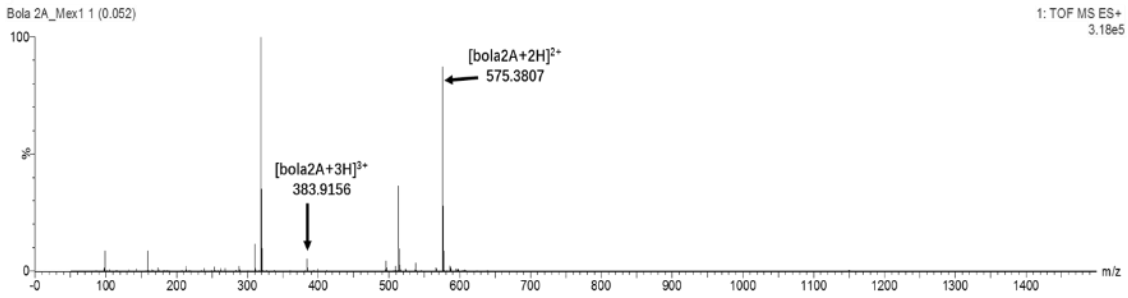
**Fig. S2.** $^{1}$H-, $^{13}$C-, $^{19}$F-NMR, ESI-HRMS and HPLC spectra of **bola2A** (A, B, C, D, E), **bola4A** (F, G, H, I, J) and **bola8A** (K, L, M, N, O).



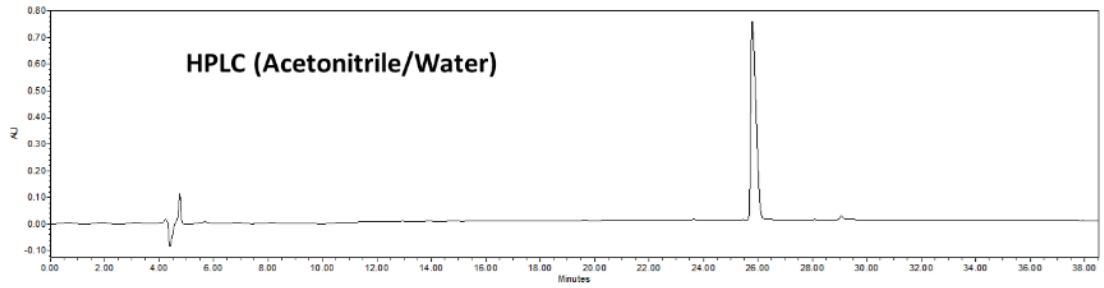
**C**


$^{19}$F-NMR, 376 MHz, $CDCl_3$
−70
−74
−78
−82
−86
−90
−94
−98
−102
−106
−110
−114
−118
−122
f1 (ppm)

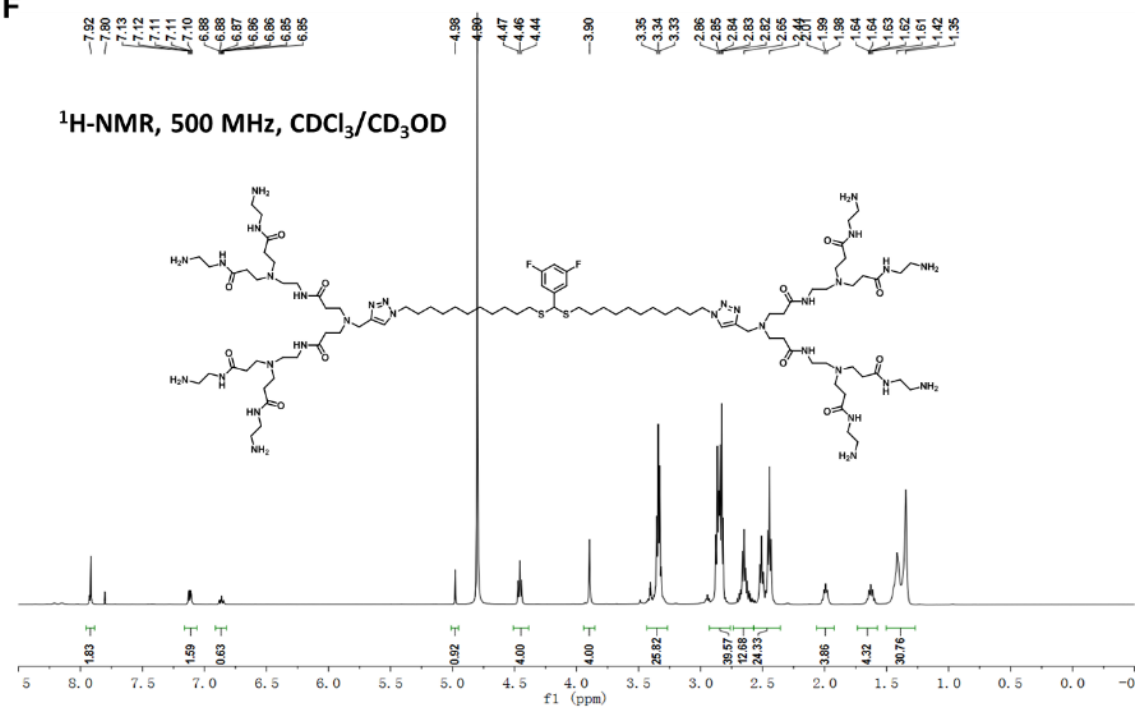
**D**


Bola 2A_Mex1 1 (0.052)
1: TOF MS ES+
3.18e5
100
%
0
[bola2A+2H]$^{2+}$
575.3807
[bola2A+3H]$^{3+}$
383.9156
-0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
m/z

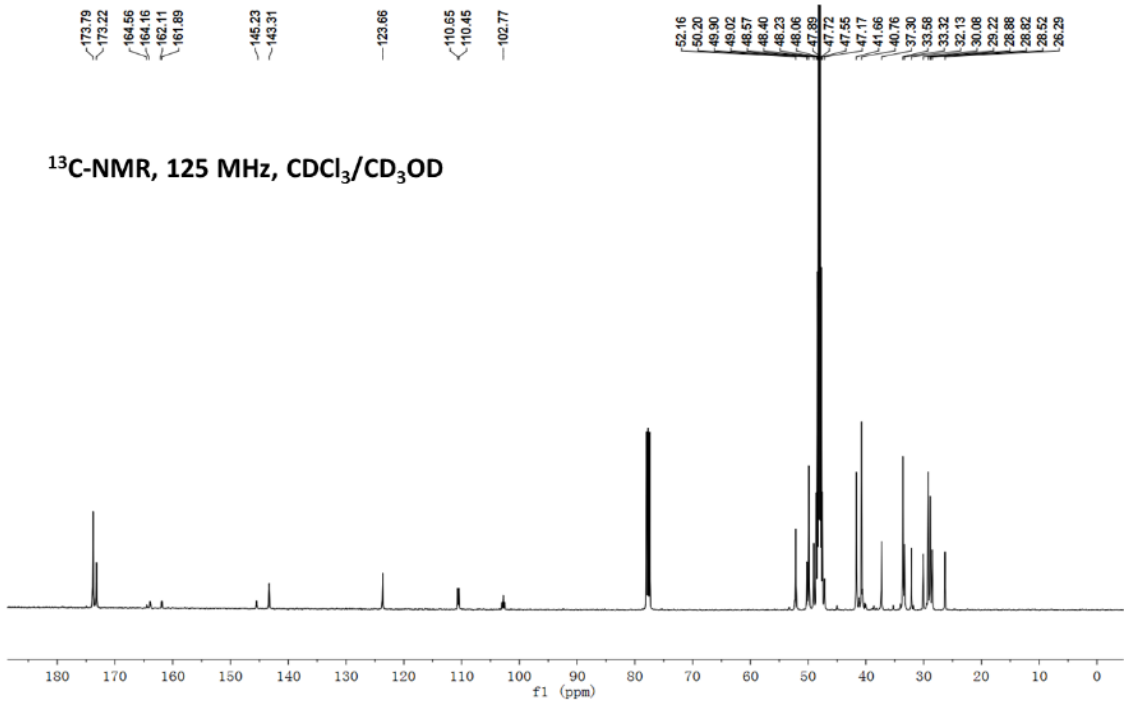
**E**


HPLC (Acetonitrile/Water)
AU
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
32.00
34.00
36.00
38.00
Minutes

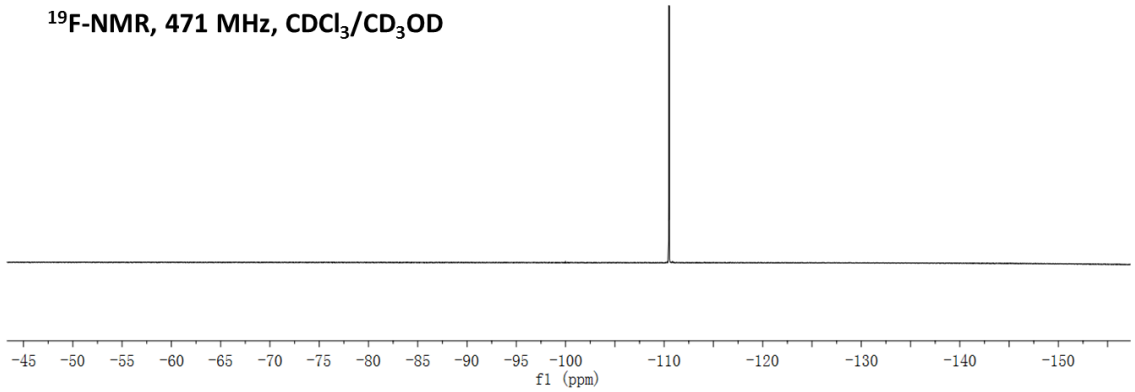
F


7.92
7.80
7.13
7.12
7.11
7.11
7.10
6.88
6.88
6.87
6.86
6.86
6.85
6.85
4.98
4.80
4.47
4.46
4.44
3.90
3.35
3.34
3.33
2.86
2.85
2.84
2.83
2.82
2.65
2.01
1.99
1.98
1.64
1.64
1.63
1.62
1.61
1.42
1.35
1H-NMR, 500 MHz, CDCl3/CD3OD
1.83
1.59
0.63
0.92
4.00
4.00
25.82
39.57
12.68
24.33
3.86
4.32
30.76
f1 (ppm)

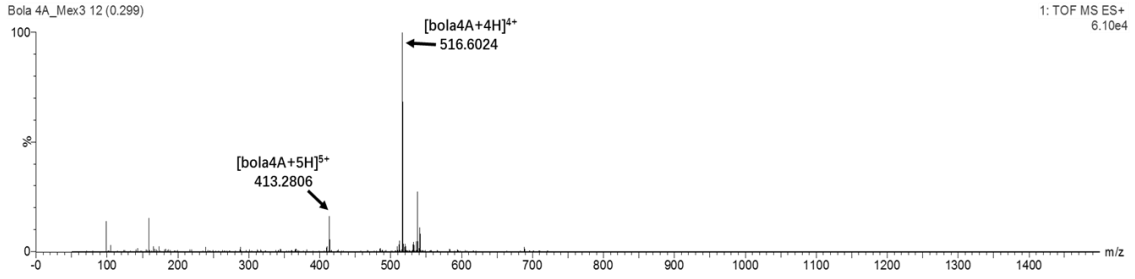
G


173.79
173.22
164.56
164.16
162.11
161.89
145.23
143.31
123.66
110.65
110.45
102.77
52.16
50.20
49.90
49.02
48.57
48.40
48.23
48.06
47.89
47.72
47.55
47.17
41.66
40.76
37.30
33.58
33.32
32.13
30.08
29.22
28.88
28.82
28.52
26.29
13C-NMR, 125 MHz, CDCl3/CD3OD
f1 (ppm)

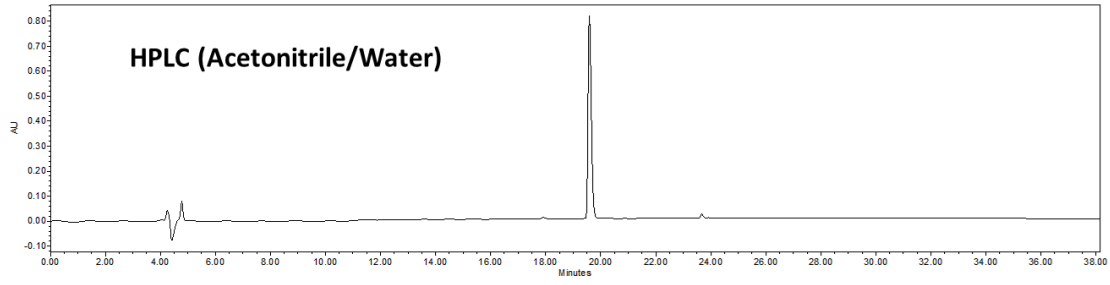
**H**


$^{19}$F-NMR, 471 MHz, $CDCl_3/CD_3OD$
-45 -50 -55 -60 -65 -70 -75 -80 -85 -90 -95 -100 -110 -120 -130 -140 -150
f1 (ppm)

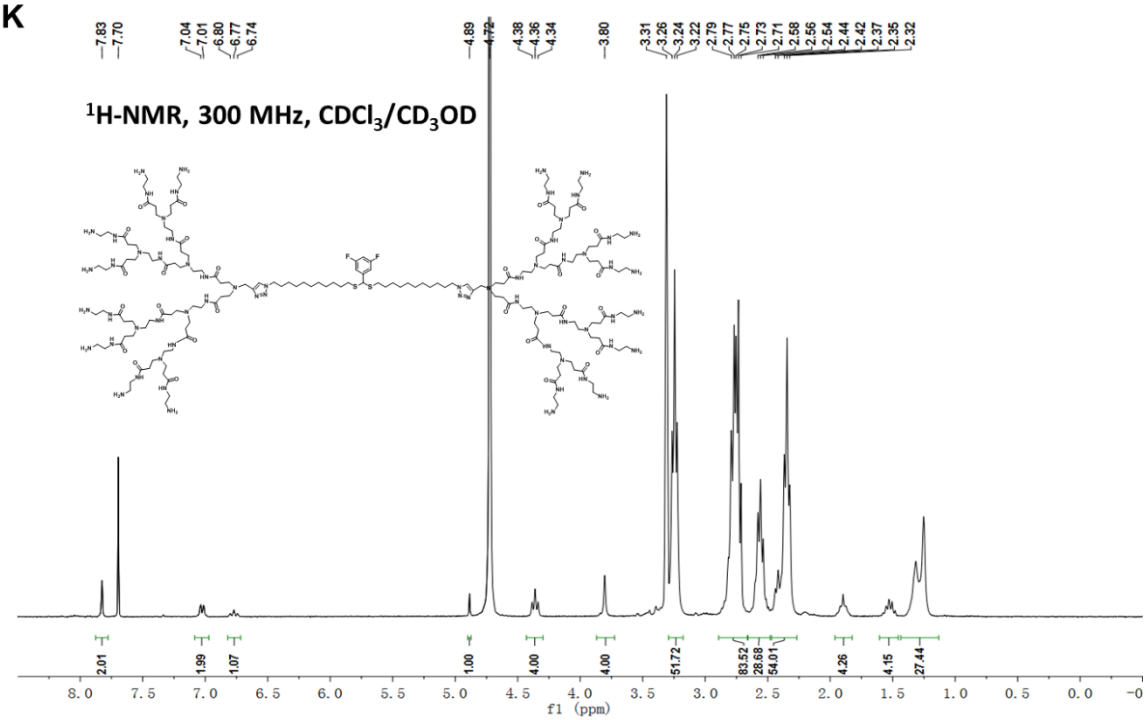
**I**


Bola 4A_Mex3 12 (0.299)
1: TOF MS ES+
6.10e4
[bola4A+4H]4+
516.6024
[bola4A+5H]5+
413.2806
%
m/z
-0 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400

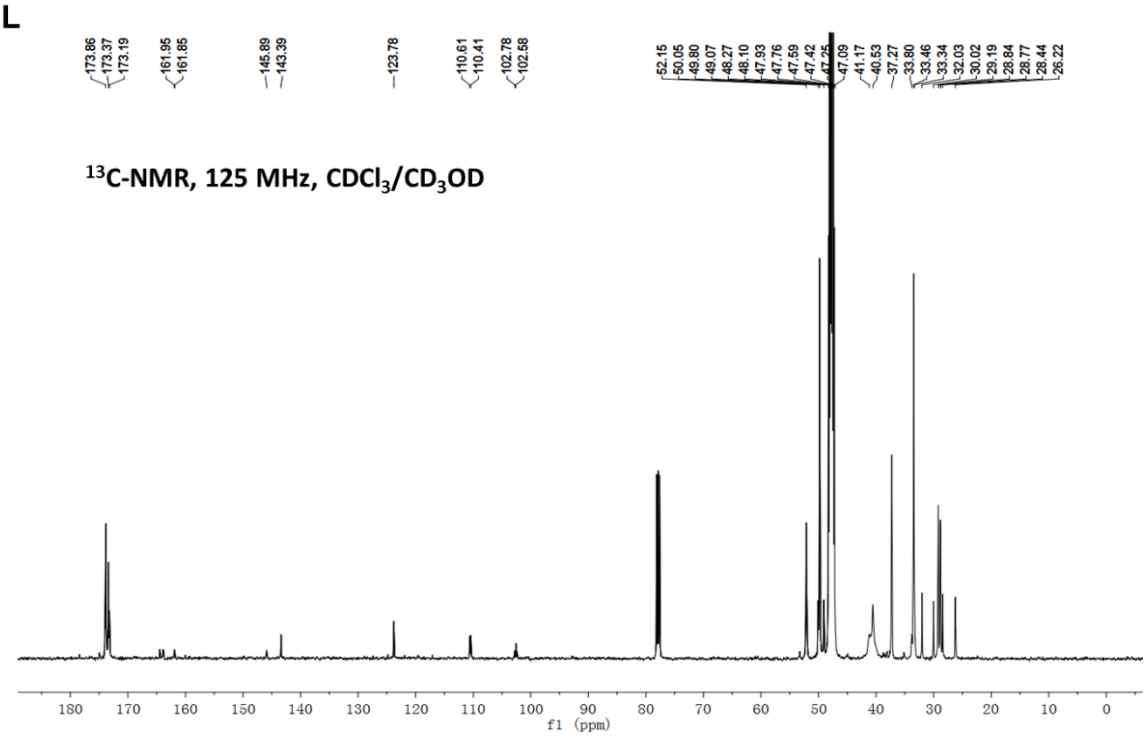
**J**


HPLC (Acetonitrile/Water)
AU
Minutes
0.00 2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00 26.00 28.00 30.00 32.00 34.00 36.00 38.00

**K**

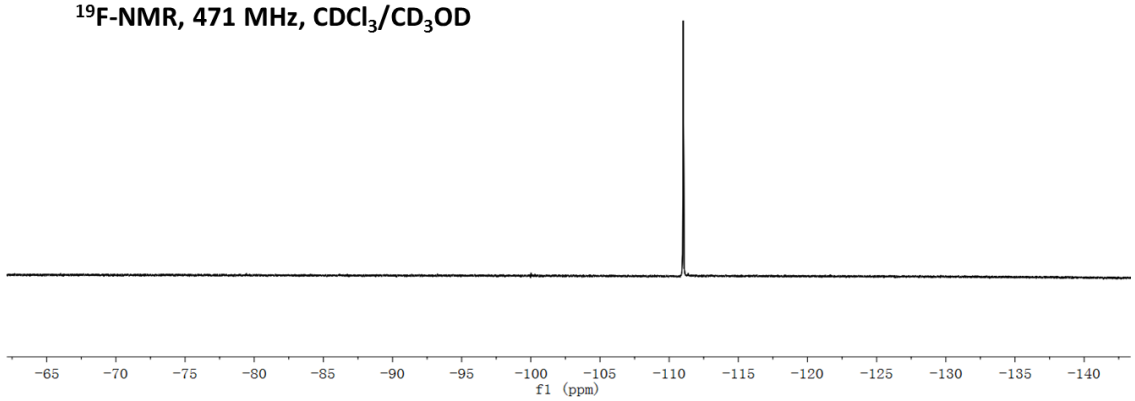

7.83
7.70
7.04
7.01
6.80
6.77
6.74
4.89
4.72
4.38
4.36
4.34
3.80
3.31
3.26
3.24
3.22
2.79
2.77
2.75
2.73
2.71
2.58
2.56
2.54
2.44
2.42
2.37
2.35
2.32
2.01
1.99
1.07
1.00
4.00
4.00
51.72
83.52
28.68
54.01
4.26
4.15
27.44
f1 (ppm)

**L**

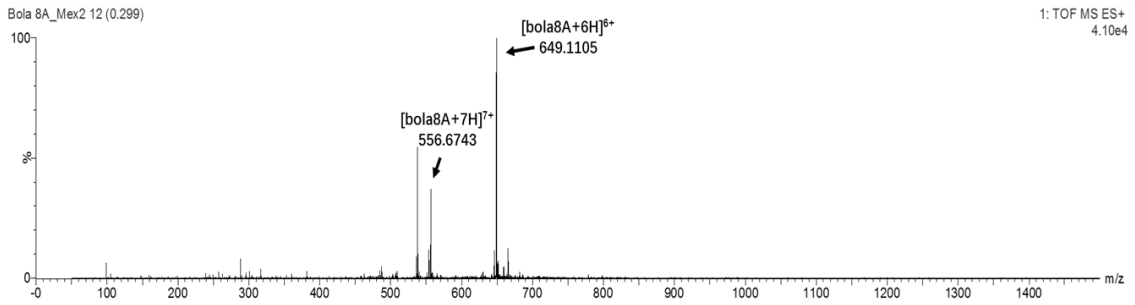

173.86
173.37
173.19
161.95
161.85
145.89
143.39
123.78
110.61
110.41
102.78
102.58
52.15
50.05
49.80
49.07
48.27
48.10
47.93
47.76
47.59
47.42
47.25
47.09
41.17
40.53
37.27
33.80
33.46
33.34
32.03
30.02
29.19
28.84
28.77
28.44
26.22
f1 (ppm)

**M**

**$^{19}$F-NMR, 471 MHz, $CDCl_3/CD_3OD$**




**N**




**O**

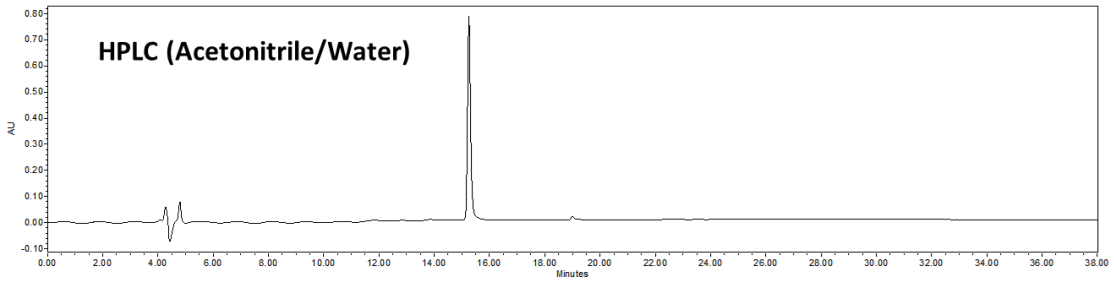


**Fig. S3. Safety evaluation of bola-amphiphilic dendrimers using *in vitro* cytotoxicity assay.** Cell viability assay of (A) HEK293 cells, (B) L929 cells, and (C) MDCK cells upon treatment with bola-amphiphilic dendrimers using PrestoBlue assay. Nontreatment was used as positive control. Membrane damage evaluation of (D) HEK293 cells, (E) L929 cells, and (F) MDCK cells upon treatment with bola-amphiphilic dendrimers using LDH assay. Lysis buffer provided the commercial LDH assay kit was used as positive control. (G) Hemolytic toxicity evaluation of bola-amphiphilic dendrimers using mouse red blood cells. Quantitative analysis of hemolysis determined by UV absorption at 540 nm. Triton was used positive control. (mean ± SD, n = 3).

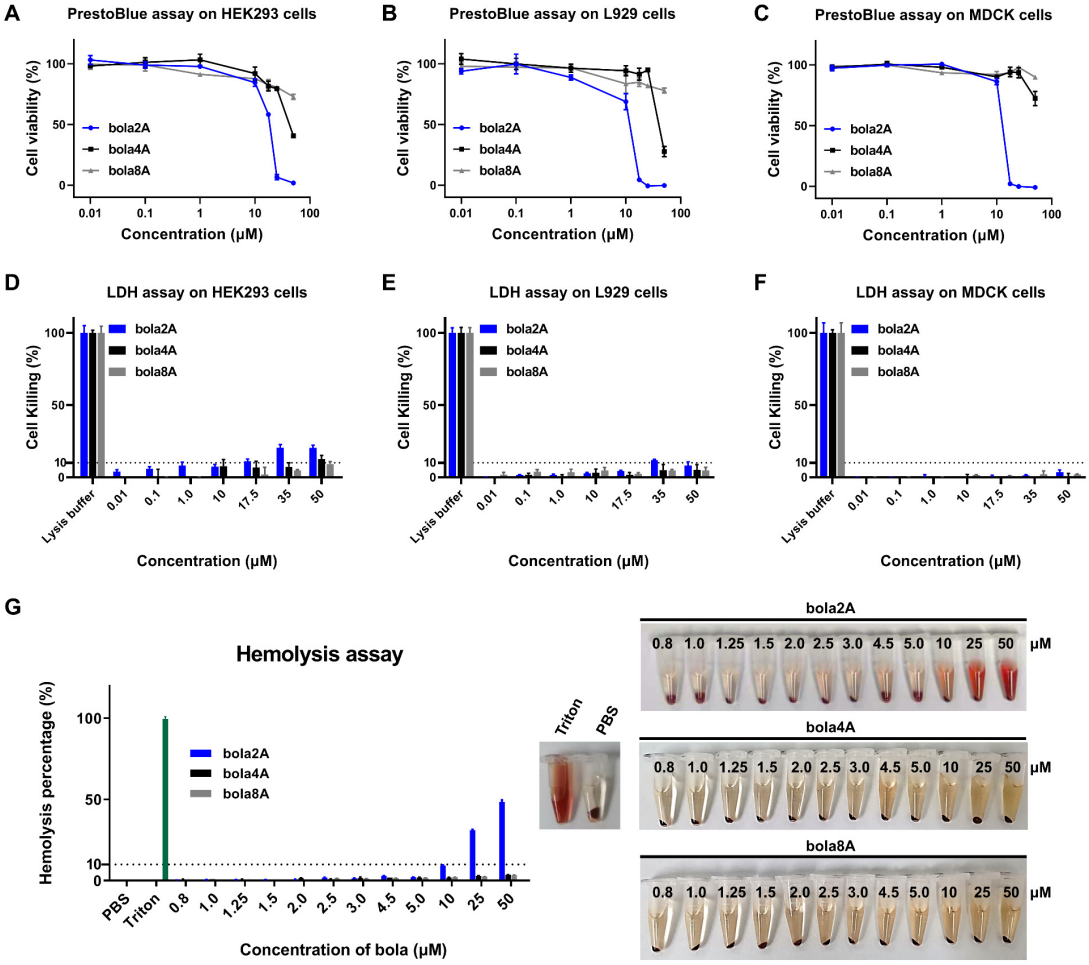


**Fig. S4. GFP protein expression upon the delivery of EGFP plasmid DNA mediated by the bola-amphiphilic dendrimers bola4A and bola8A.** GFP protein expression in different cell lines was assessed using flow cytometry: (A) SKOV-3 cells (12 ng/μL DNA, N/P ratio of 1.0), (B) HeLa cells (4.0 ng/μL DNA, N/P ratio of 1.0) and (C) PC-3 cells (4 ng/μL DNA at N/P ratio of 1.0). The commercial transfection reagent Lipofectamine 2000 (Lipo) was used as positive control. pEGFP: plasmid DNA expressing GFP. (mean ± SD, n = 3).

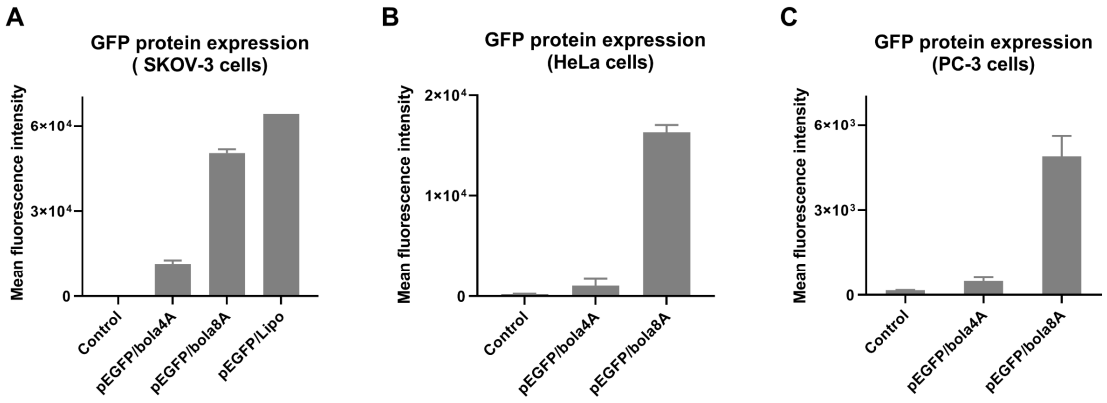


**Fig. S5. GFP protein expression upon the delivery of EGFP siRNA mediated by the bola-amphiphilic dendrimers bola4A and bola8A in HeLa-GFP cells.** (A) Fluorescent imaging and (B) flow cytometry analysis of GFP protein expression following treatment with PBS control, **bola4A** alone, **bola8A** alone, siEGFP/**bola4A**, scramble/**bola4A**, siEGFP/Lipo, siEGFP/**bola8A**, scramble/**bola8A** (50 nM siRNA, N/P ratio of 10). The commercial transfection reagent Lipofectamine 2000 (Lipo) was used as positive control. siEGFP: siRNA targeting GFP, scramble: scramble siRNA. Scale bar, 200 μm.

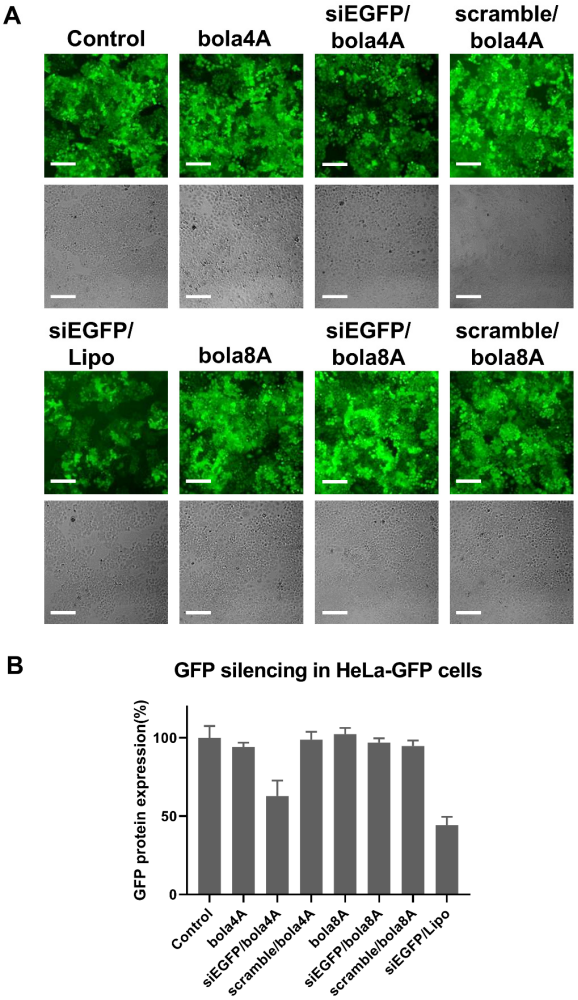


**Fig. S6. Bola-amphiphilic dendrimer mediated delivery of siRNAs targeting either AKT2 or HSP27 for gene silencing in different cell lines.** AKT2 and HSP27 protein expression in different cells was assessed using Western blotting, following treatment with bola-amphiphilic dendrimers **bola4A** and **bola8A** alone, siRNA alone, siAKT2/dendrimer complexes, siHSP27/dendrimer complexes, scramble/dendrimer complexes (50 nM siRNA, N/P ratio of 10). AKT2 protein expression in (A) SKOV-3 cells and (B) PANC-1 cells, and HSP27 protein expression in (C) SKOV-3 cells and (D) PC-3 cells. siAKT2: siRNA targeting AKT2, siHSP27: siRNA targeting HSP27, scramble: scramble siRNA. Quantitative analysis of western blotting determined by Image J software. (mean ± SD, n = 3).

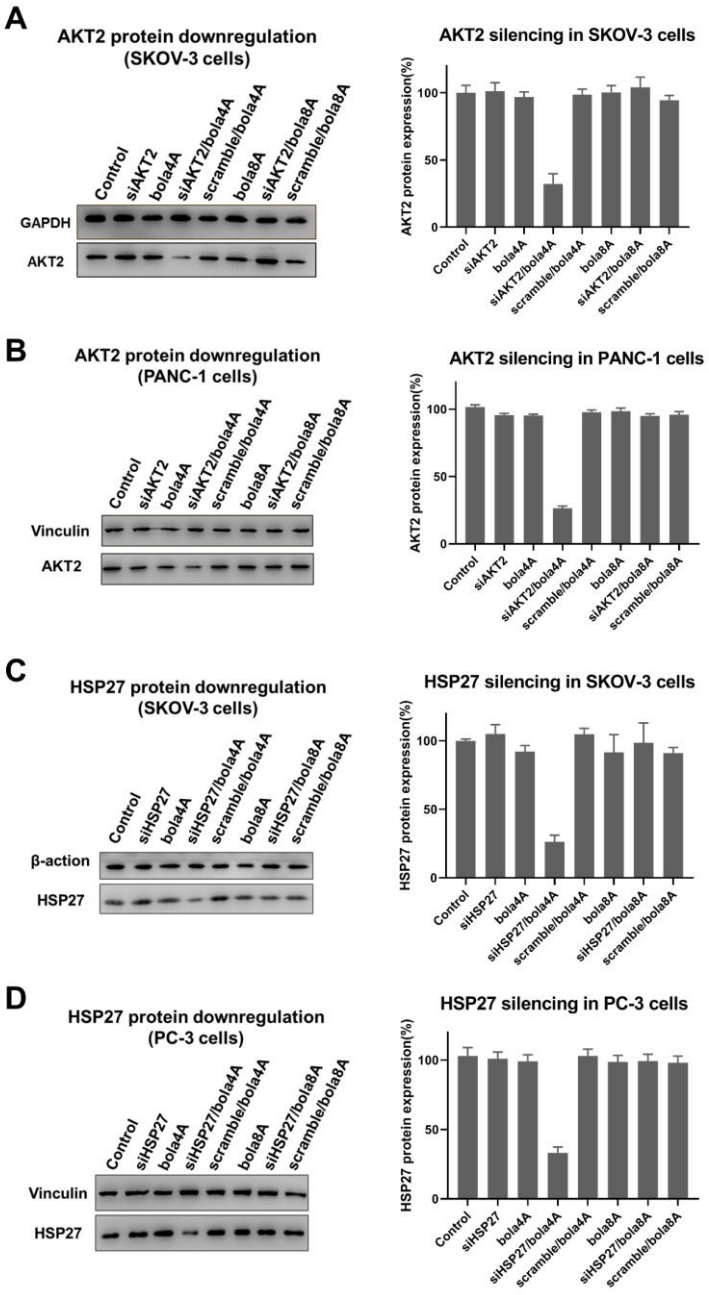


**Fig. S7. Safety assessment of bola-amphiphilic dendrimer mediated nucleic acid delivery using *in vitro* cytotoxicity assays.** MTT assay of the metabolic toxicity of (A) the DNA/dendrimer and (B) the siRNA/dendrimer complexes formed with the bola-amphiphilic dendrimers **bola4A** and **bola8A**, respectively. LDH assay of the membrane damage toxicity of (C) the DNA/dendrimer complexes and (D) the siRNA/dendrimer complexes formed with the bola-amphiphilic dendrimers **bola4A** and **bola8A**. The control cells were treated with lysis buffer as 100% LDH release. (E) Hemolysis assay of the DNA- and siRNA-complexes formed with the bola-amphiphilic dendrimers **bola4A** and **bola8A**. Quantitative analysis of hemolysis was performed by recording UV absorption at 540 nm. scramble: scramble siRNA. (mean ± SD, n = 3). Nontreatment was used as positive control for MTT assay. Lysis buffer provided the commercial LDH assay kit was used as positive control for LDH assay. Triton was used positive control for hemolysis assay.

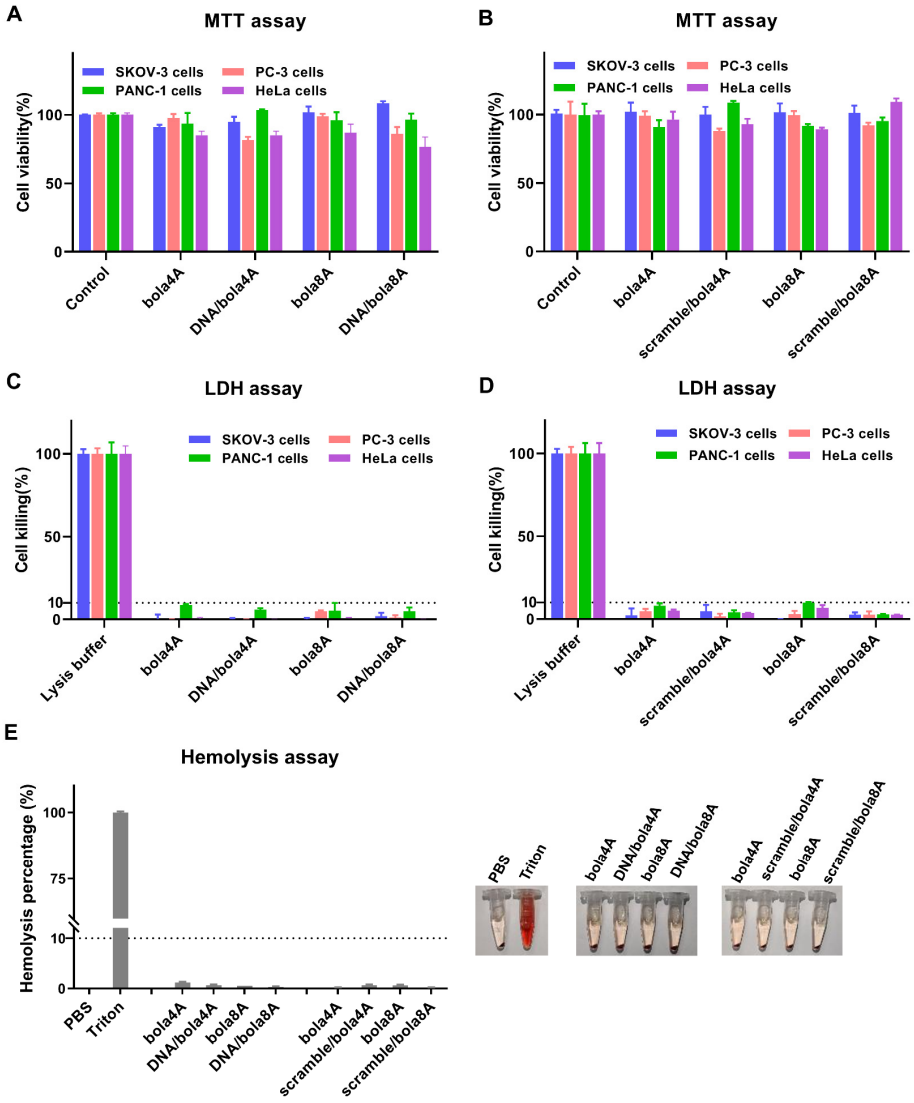


**Fig. S8. Cell uptake and intracellular trafficking of nucleic acid/dendrimer complexes.** (A) Cellular uptake of the complexes of DNA/**bola4A** and DNA/**Bola8A** in SKOV-3 cells was quantified at different time point post-treatment using flow cytometry (12 ng/μL YOYO-1 labeled DNA, N/P ratio of 1.0). $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$ versus DNA/**bola4A** or DNA/**bola8A** and significance was determined using two-way ANOVA. (B) Cellular uptake kinetics of the complexes of siRNA/**bola4A** and siRNA/**Bola8A** in SKOV-3 cells was assessed at different time point post-treatment using flow cytometry (50 nM Cy5-labeled siRNA, N/P ratio of 10). (C) Confocal imaging of the endosomal escape of the siRNA/dendrimer complexes in SKOV-3 cells (50 nM Cy5-labeled siRNA, N/P ratio of 10). The red channel image shows the Cy5-labeled siRNA (red), the green channel image shows the lysosome stained by lysotracker (green), and the blue channel image shows the nuclei of SKOV-3 cells stained by Hoechst33342 (blue). (mean ± SD, n = 3).

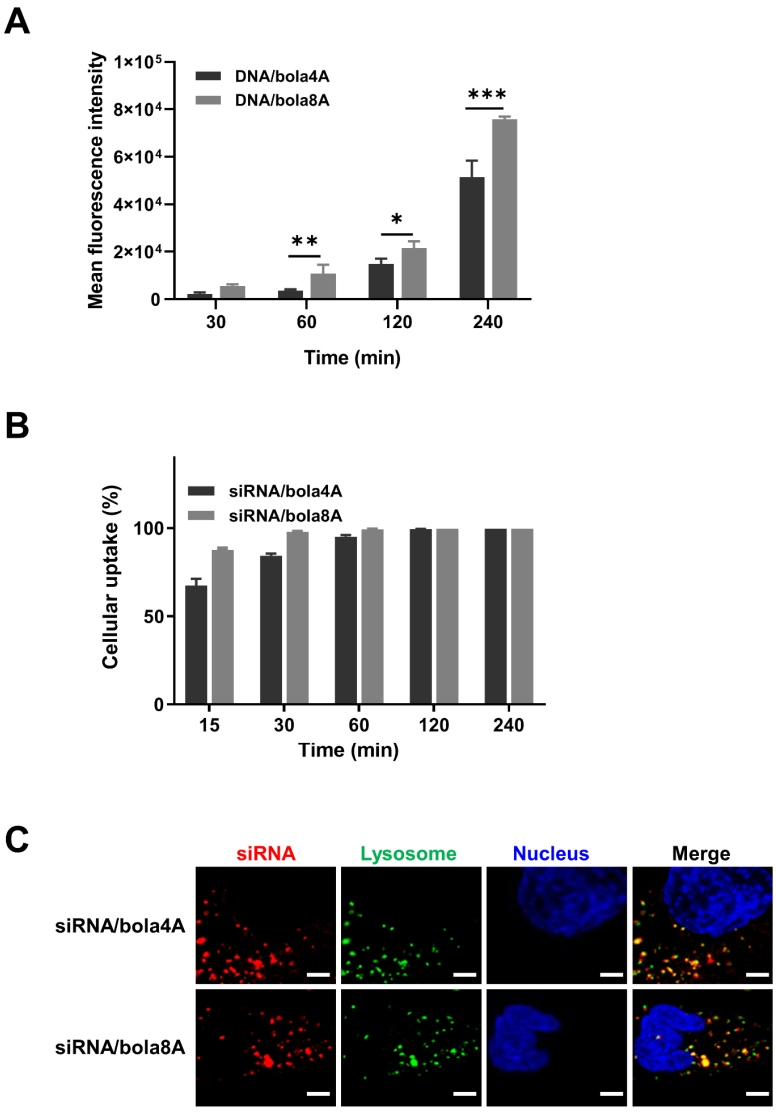


**Fig. S9. ROS-specific delivery of nucleic acid/dendrimer complexes.** (A) ROS level in SKOV-3 cells and NAC-treated SKOV-3 cells (SKOV-3 cells incubated with 10 mM NAC). *** $p \leq 0.001$, significance was determined using unpaired Student's *t* test. (B) EGFP protein expression in SKOV-3 cells and NAC-treated SKOV-3 cells following incubation with pEGFP complexed by **bola8A** (12 ng/μL DNA, N/P ratio of 1.0) using flow cytometry. *** $p \leq 0.001$, significance was determined using two-way ANOVA. pEGFP: plasmid DNA expressing GFP. (mean ± SD, n = 3).

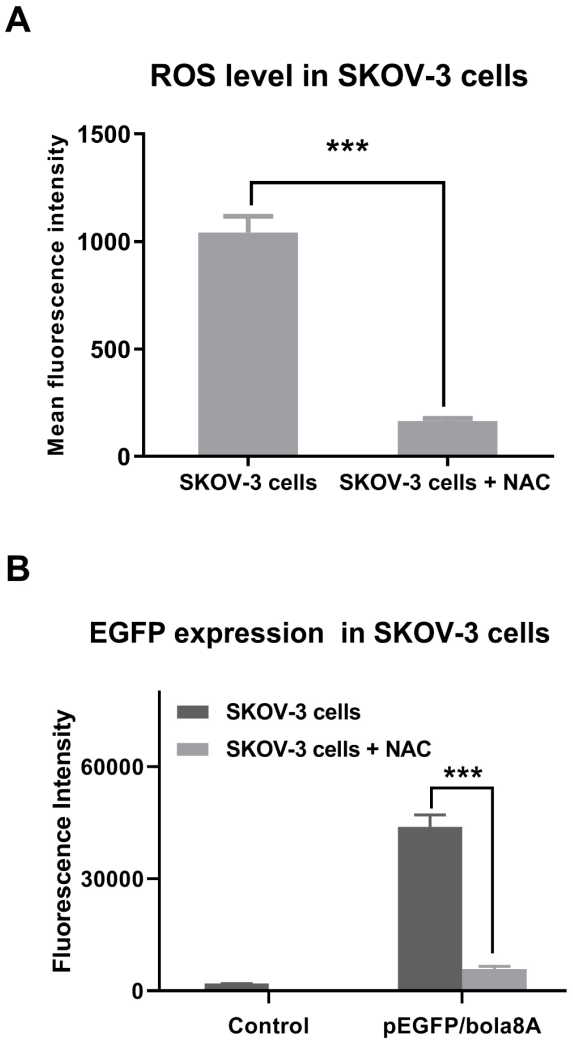


**Fig. S10. *In vivo* biodistribution of nucleic acid/dendrimer complexes in tumor-xenograft mice.** The image (A) and quantitative analysis (B) of fluorescence signals of Cy5-labeled DNA/**bola8A** in tumor and other major organs resected from SKOV-3 xenograft mice after intravenous administration (0.25 mg/kg Cy5-labeled DNA, 0.2 mg/kg **bola8A**, N/P ratio of 1.0). The image (C) and quantitative analysis (D) of Cy5-labeled siRNA/**bola4A** in tumor and other major organs resected from SKOV-3 xenograft mice after intravenous administration (1.0 mg/kg Cy5-labeled siRNA, 3.9 mg/kg **bola4A**, N/P ratio of 5.0).

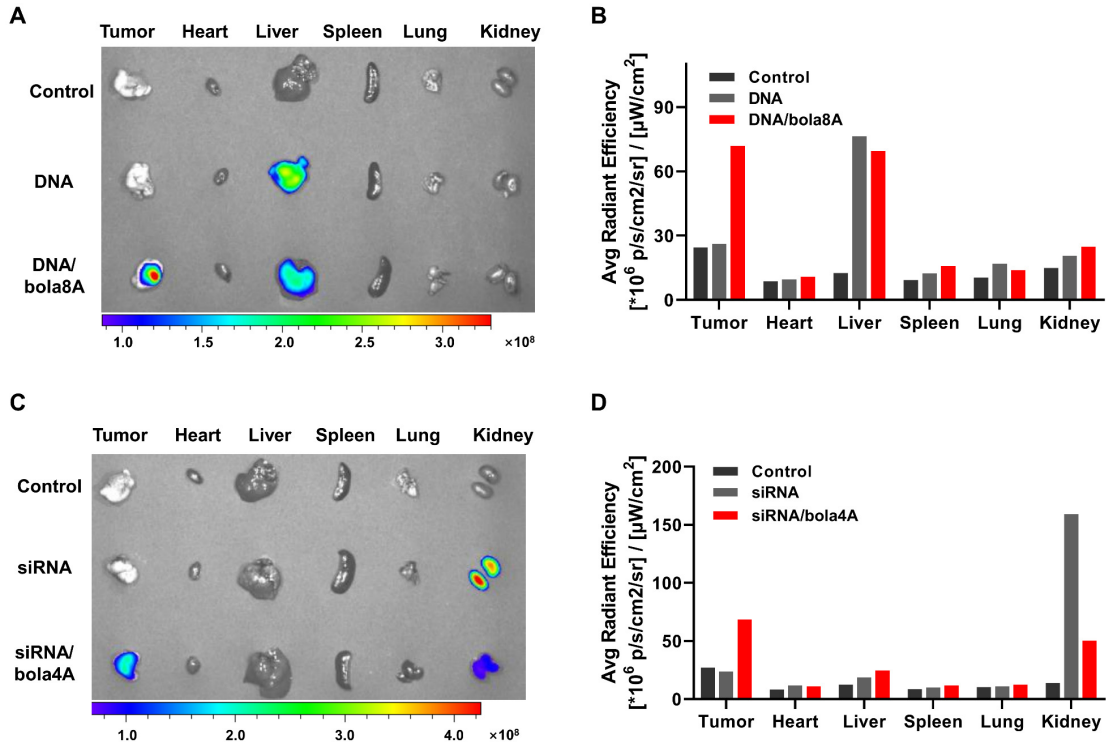


**Fig. S11. Bola-amphiphilic dendrimer bola8A mediated delivery of p53 plasmid DNA for gene transfection and cancer treatment in cervical cancer model.** Treatment schedule for cervical tumor model: BALB/c nude mice were inoculated with HeLa cells 14 days before treatment. HeLa xenograft mice were intravenously administrated with PBS buffer (control), **bola8A** alone, p53 alone, p53/**bola8A** complex (1.0 mg/kg DNA, 0.7 mg/kg **bola8A**, N/P ratio of 1.0) twice per week for two weeks. (A) Tumor weight of the resected HeLa tumors after sacrifice of mice. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, significance was determined using one-way ANOVA. (B) Western blotting analysis of p53 protein expression in HeLa xenograft mice. (C) Cell apoptosis detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay in HeLa tumors. (D) Body weights of mice bearing HeLa tumors were measured at different time-points during the treatment regimes. (E) Blood biochemistry analysis of liver and kidney function and (F) histological analysis of tissues from major organs of HeLa xenograft mice at the end point. p53: plasmid DNA expressing tumor suppressor protein p53. Scale bar, 200 μm. (mean ± SD, n = 3).

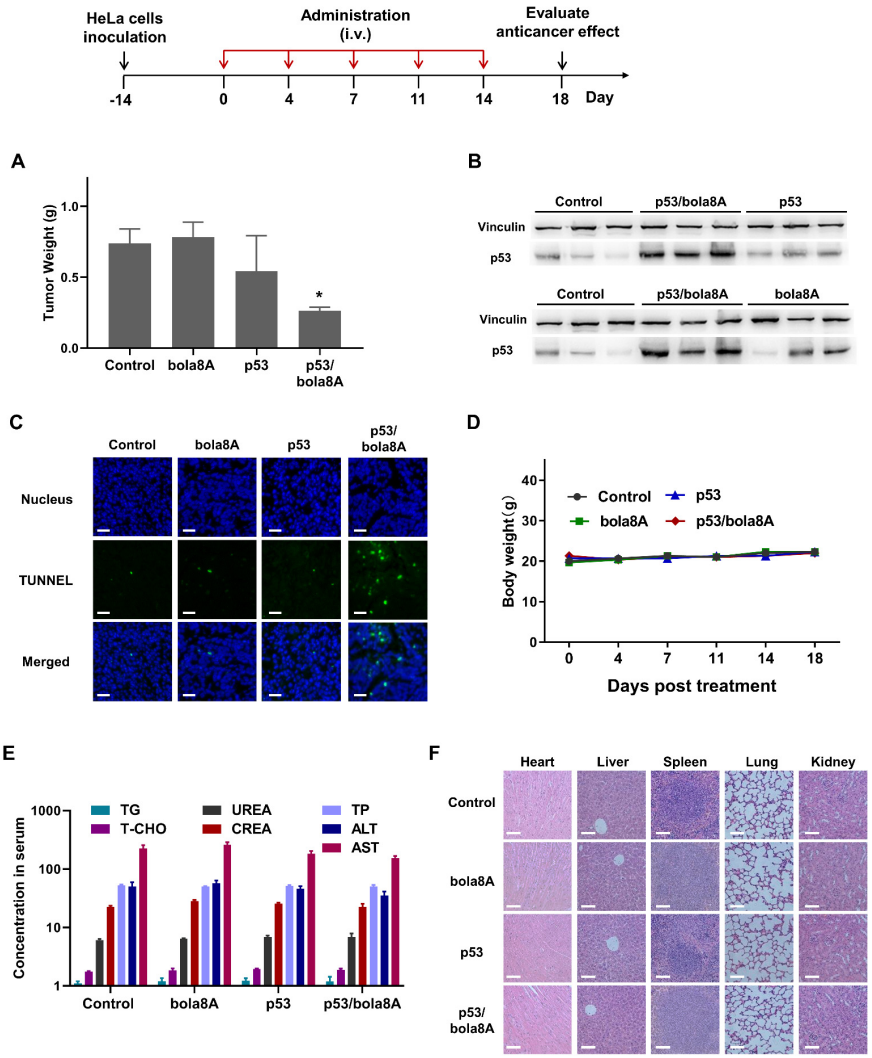


**Fig. S12. Bola-amphiphilic dendrimer bola4A mediated delivery of AKT2 siRNA for gene silencing and cancer treatment in ovarian cancer model.** Treatment schedule for ovarian tumor model: BALB/c nude mice were inoculated with SKOV-3 cells 21 days before treatment. SKOV-3 xenograft mice were intravenously administrated with PBS buffer (control), siAKT2 alone, **bola4A** alone, scramble/**bola4A** complex, siAKT2/**bola4A** complex (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio of 5.0) twice per week for two weeks. (A) Tumor weight of the resected SKOV-3 tumors after sacrifice of mice $^{**}$ $p \leq 0.01$, $^{**}$ $p \leq 0.001$, significance was determined using one-way ANOVA. (B) Western blotting analysis of AKT2 protein expression and (C) cell apoptosis detected using the TUNEL assay in resected tumors from SKOV-3 xenograft mice after treatment. (D) Body weights of SKOV-3 xenograft mice were recorded at different time-points during the treatment regimes. (E) Blood biochemistry analysis of liver and kidney function and (F) histological analysis of tissues from major organs of SKOV-3 xenograft mice after treatment. siAKT2: siRNA targeting AKT2, scramble: scramble siRNA. Scale bar, 200 μm. (mean ± SD, n = 3).

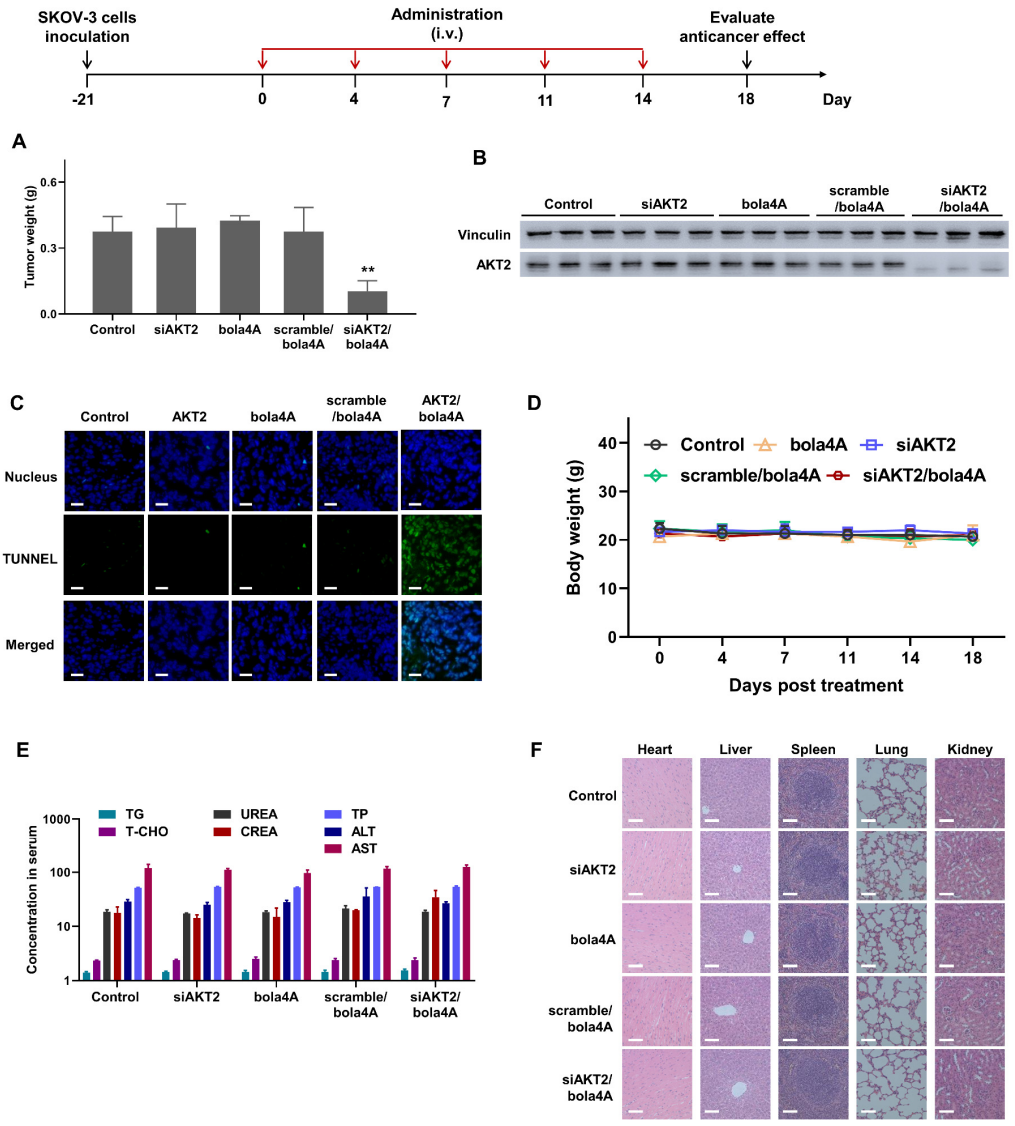

SKOV-3 cells inoculation
Administration (i.v.)
Evaluate anticancer effect
-21
0
4
7
11
14
18
Day
A
Tumor weight (g)
0.6
0.3
0.0
**
Control
siAKT2
bola4A
scramble/ bola4A
siAKT2/ bola4A
B
Control
siAKT2
bola4A
scramble /bola4A
siAKT2 /bola4A
Vinculin
AKT2
C
Control
AKT2
bola4A
scramble /bola4A
AKT2/ bola4A
Nucleus
TUNNEL
Merged
D
Body weight (g)
40
30
20
10
0
Control
bola4A
siAKT2
scramble/bola4A
siAKT2/bola4A
0
4
7
11
14
18
Days post treatment
E
Concentration in serum
1000
100
10
1
TG
T-CHO
UREA
CREA
TP
ALT
AST
Control
siAKT2
bola4A
scramble/ bola4A
siAKT2/ bola4A
F
Heart
Liver
Spleen
Lung
Kidney
Control
siAKT2
bola4A
scramble/ bola4A
siAKT2/ bola4A

**Fig. S13**. **Bola-amphiphilic dendrimers mediated nucleic acid delivery and the resulting inhibition on tumor metastasis in lung cancer metastasis model.** Treatment schedule for lung metastatic breast cancer model: after intravenous injection of 4T1-luc cells in BALB/c mice 1 day before treatment, the mice were intravenously administrated with PBS buffer (control), p53 alone, p53/**bola8A** (2.0 mg/kg DNA, 1.5 mg/kg **bola8A**, N/P ratio of 1.0), p53/Lipo (2.0 mg/kg DNA), scramble/**bola4A**, siAKT2/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio of 5.0), siAKT2/MC3 (1.0 mg/kg siRNA, N/P ratio of 5) every other day from day 0 to 10. Quantitative analysis of (A) in vivo bioluminescent signals of the tumor metastases in the 4T1-luc metastatic tumor-bearing mice and (B) ex vivo bioluminescent signals of the 4T1-luc tumor metastases in lung (day 12). Treatment schedule for lung metastatic melanoma model using **bola8A** for p53 delivery: after intravenous injection of B16-F10-luc cells in C57BL6 mice 1 day before treatment, the mice were intravenously administrated with PBS buffer (control), p53 alone, p53/**bola8A** (2.0 mg/kg DNA, 1.5 mg/kg **bola8A**, N/P ratio of 1.0), p53/JetPEI (2.0 mg/kg DNA) every other day from day 0 to 10. Quantitative analysis of (C) in vivo bioluminescent signals of the tumor metastases in the B16-F10-luc metastatic tumor-bearing mice and (D) ex vivo bioluminescent signals of the B16-F10-luc tumor metastases in lung (day 12). Treatment schedule for lung metastatic melanoma model using **bola4A** for siAKT2 delivery: after intravenous injection of B16-F10-luc cells in C57BL6 mice 1 day before treatment, the mice were intravenously administrated with PBS buffer (control), scramble/**bola4A**, siAKT2/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio of 5.0), siAKT2/MC3 (1.0 mg/kg siRNA, N/P ratio of 5) every other day from day 0 to 6. Quantitative analysis of (E) in vivo bioluminescent signals of the tumor metastases in the B16-F10-luc metastatic tumor-bearing mice and (F) ex vivo bioluminescent signals of the B16-F10-luc tumor metastases in lung (day 8). Quantitative analysis of histological images of the lung tissues from (G) the 4T1-luc metastatic tumor-bearing mice and (H) (I) the B16-F10-luc tumor-bearing mice. The data were collected at the experimental end point. ns: not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, significance was determined using Mann-Whitney test. (mean ± SD, n =5). p53: plasmid DNA expressing tumor suppressor protein p53, siAKT2: siRNA targeting AKT2, scramble: scramble siRNA, Lipo: Lipofectamine 2000, MC3: DLin-MC3-DMA (MC3), an FDA-approved LNP for siRNA delivery, JetPEI, a commercial in vivo transfection reagent for plasmid DNA.

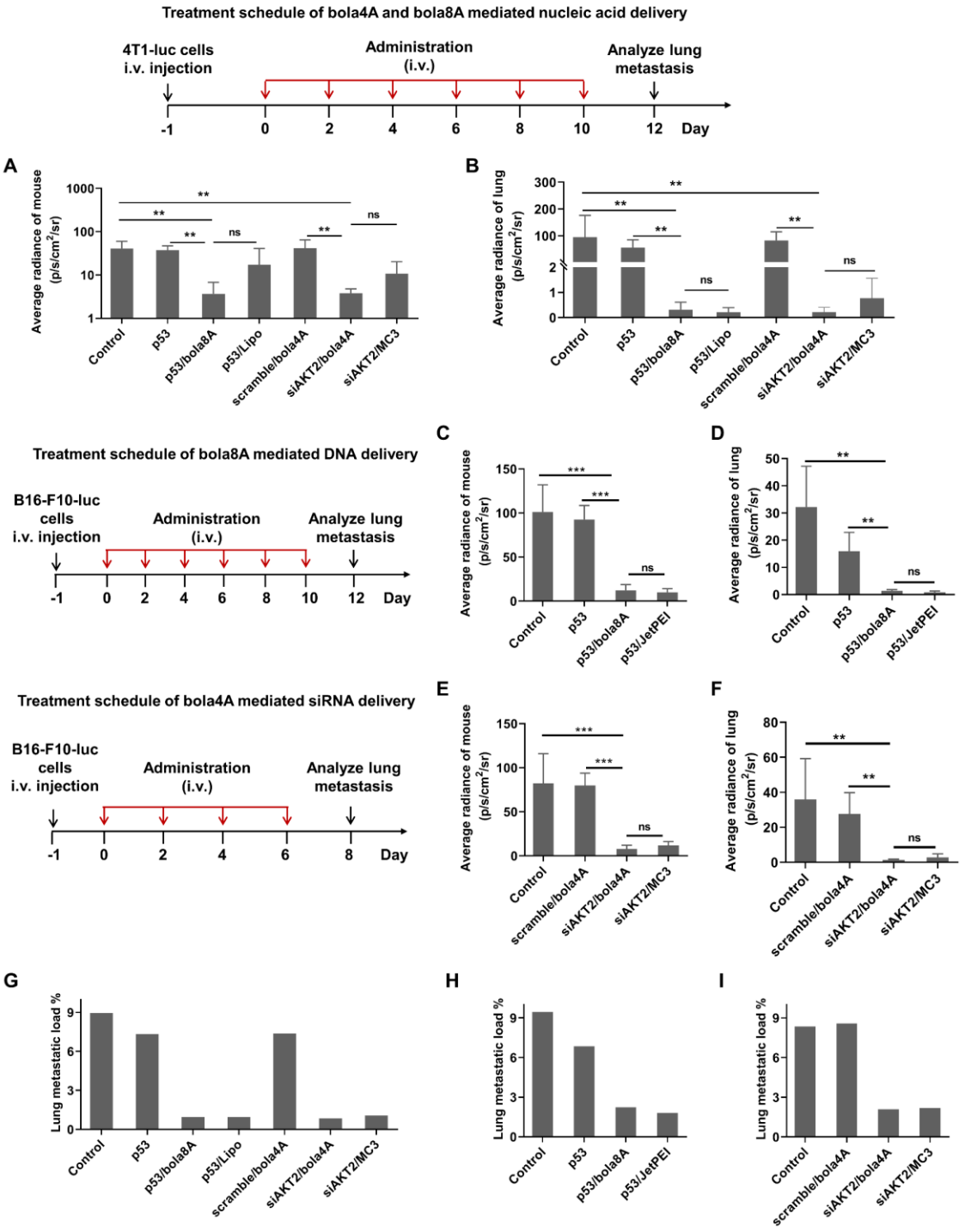

Treatment schedule of bola4A and bola8A mediated nucleic acid delivery
4T1-luc cells i.v. injection
Administration (i.v.)
Analyze lung metastasis
-1 0 2 4 6 8 10 12 Day
A
Average radiance of mouse (p/s/cm²/sr)
Control p53 p53/bola8A p53/Lipo scramble/bola4A siAKT2/bola4A siAKT2/MC3
B
Average radiance of lung (p/s/cm²/sr)
Control p53 p53/bola8A p53/Lipo scramble/bola4A siAKT2/bola4A siAKT2/MC3
Treatment schedule of bola8A mediated DNA delivery
B16-F10-luc cells i.v. injection
Administration (i.v.)
Analyze lung metastasis
-1 0 2 4 6 8 10 12 Day
C
Average radiance of mouse (p/s/cm²/sr)
Control p53 p53/bola8A p53/JetPEI
D
Average radiance of lung (p/s/cm²/sr)
Control p53 p53/bola8A p53/JetPEI
Treatment schedule of bola4A mediated siRNA delivery
B16-F10-luc cells i.v. injection
Administration (i.v.)
Analyze lung metastasis
-1 0 2 4 6 8 Day
E
Average radiance of mouse (p/s/cm²/sr)
Control scramble/bola4A siAKT2/bola4A siAKT2/MC3
F
Average radiance of lung (p/s/cm²/sr)
Control scramble/bola4A siAKT2/bola4A siAKT2/MC3
G
Lung metastatic load %
Control p53 p53/bola8A p53/Lipo scramble/bola4A siAKT2/bola4A siAKT2/MC3
H
Lung metastatic load %
Control p53 p53/bola8A p53/JetPEI
I
Lung metastatic load %
Control scramble/bola4A siAKT2/bola4A siAKT2/MC3

**Fig. S14. Effective inhibition on tumor metastasis using therapeutic DNA p53 delivered by bola8A and JetPEI in 4T1-luc lung metastasis mouse model.** Treatment schedule for lung metastasis after intravenous 4T1-luc cells injection: the mice were intravenously administrated with PBS buffer (control), p53 alone, p53/**bola8A** (2.0 mg/kg DNA, 1.5 mg/kg **bola8A**, N/P ratio of 1.0), p53/JetPEI (2.0 mg/kg DNA, N/P ratio of 1.0) every two days from days 0 to 10. In vivo bioluminescence imaging (A) and quantitative analysis (B) of 4T1-luc tumor metastases in mice. Ex vivo bioluminescence imaging (C) and quantitative analysis (D) of 4T1-luc tumor metastases in the lung at the experimental end point post treatment. (E) Histological analysis of lung tissues from 4T1-luc metastatic tumor-bearing mice at the experimental end point post treatment. The metastatic lesions (red solid outlines) were identified as cell clusters with darkly stained nuclei. (F) Quantitative analysis of histological images of the lung tissues from the 4T1-luc metastatic tumor-bearing mice. (G) p53 protein expression revealed using immunohistochemistry staining after treatments. Data were collected at the experimental end point (day 12). ns: not significant, $^{**}p \leq 0.01$, significance was determined using Mann-Whitney test. (mean ± SD, n =5). p53: plasmid DNA expressing tumor suppressor protein p53; JetPEI, a commercial in vivo transfection reagent for plasmid DNA.

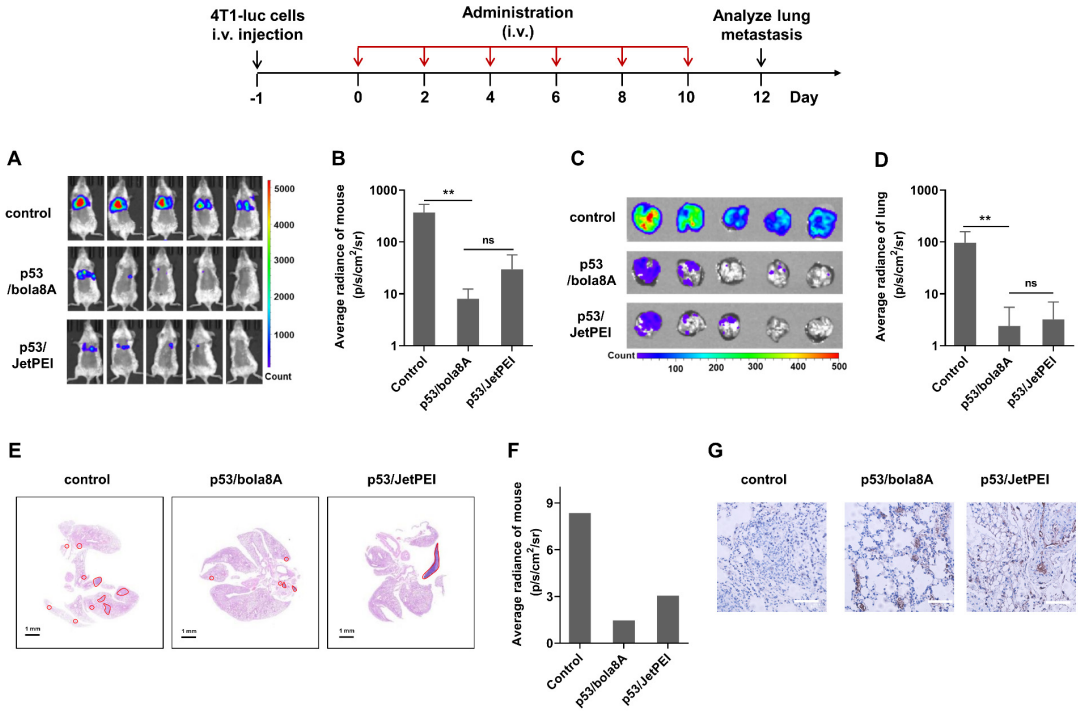
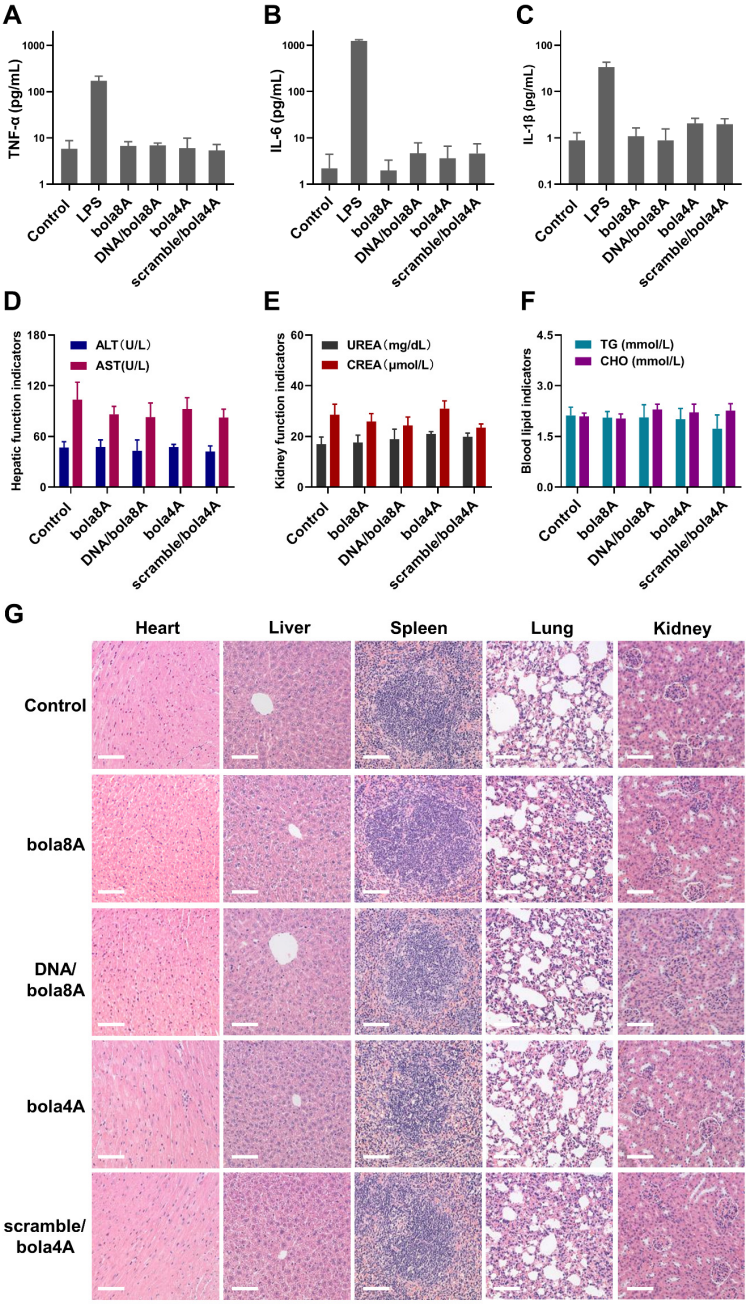


**Fig. S15. Toxicity assessment of bola-amphiphilic dendrimers and their corresponding complexes with nucleic acids in healthy mice.** Inflammatory response by quantifying the serum levels of the representative major cytokines (A) TNF-α, (B) IL-6, and (C) IL-1β; Liver and kidney function as well as blood lipid by quantifying the levels of biomarkers (D) ALT and AST, (E) UREA and CREA, (F) TG and CHO in serum. $^{***}p \leq 0.001$, significance was determined using two-way ANOVA (mean ± SD, n = 3). (G) Histological analysis of tissues from major organs. Mice were administrated intravenously with PBS, **bola8A** alone, DNA/**bola8A** (1.0 mg/kg DNA, 0.7 mg/kg **bola8A**, N/P ratio of 1.0), **bola4A** alone, scramble/**bola4A** (3.0 mg/kg siRNA, 11.6 mg/kg **bola4A**, N/P ratio of 5.0). scramble: scramble siRNA. Scale bar, 200 μm.



**Synthesis and characterization of bola-amphiphilic dendrimers**

All the reagents and solvents were purchased from Sigma Aldrich or Alfa Aesar. Methyl acrylate and ethylenediamine were dried according to the described methods and distilled before use. The bola-core and the dendrons 2E, 4E and 8E were synthesized following the previous protocols[1]. The other chemicals were used without further purification. Analytical thin layer chromatography (TLC) was performed using silica gel 60 $F_{254}$ plates 0.2 mm thick with UV light (254 and 364 nm) as revelator. Chromatography was prepared on silica gel (Merck 200-300 mesh). Dialysis tubing was purchased from Sigma Aldrich (St. Quentin Fallavier, France) and Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

High-performance liquid chromatography (HPLC) was performed on a Waters Empower system (Waters 1525, binary HPLC pump) equipped with a photodiode array detector (Waters 2998) and a SinoChrom C8 column (5 μm, 4.6 mm × 250 mm), using a gradient elution mode (20–70% acetonitrile in water) over 38 minutes with acetonitrile and water as the mobile phases (both containing 0.04% TFA). The flow rate was 0.8 mL/min, and the injection volume was 20 μL. Peaks were detected at 210 nm. The retention times (RTs) were in minutes.

$^{1}H$, $^{13}C$, $^{19}F$ and 2D-NMR spectra were recorded on Bruker Avance III 400 (400 MHz, $^{1}H$; 101 MHz, $^{13}C$; 376 MHz, $^{19}F$), JEOL ECS 400 spectrometers (400 MHz, $^{1}H$; 101 MHz, $^{13}C$; 376 MHz, $^{19}F$) and Bruker Avance III 500 (500 MHz, $^{1}H$; 125 MHz, $^{13}C$; 471 MHz, $^{19}F$). The temperature for all NMR data collection is 300 K if not specified. Chemical shifts (δ) are expressed in parts per million (ppm). The HRMS analysis was carried out with a SYNAPT G2 HDMS (Waters) mass spectrometer equipped with a pneumatically assisted atmospheric pressure ionization (API) source. High-resolution mass spectra (MS) were obtained with a flight time analyzer (TOF). Each accurate mass measurement was done in triplicate using an external calibration.

**Synthesis of bola2E**

To the azide precursor **core** (230 mg, 0.39 mmol) were added $CuSO_4 \cdot 5H_2O$ (20 mg, 0.078 mmol), sodium ascorbate (39 mg, 0.20 mmol) and a solution of **2E** (215 mg, 0.95

mmol) in 12 mL THF. The vessel was sealed and purged with argon for 5 min and 3.0 mL $H_2O$ was then added into the mixture. The reaction mixture was stirred at 50 °C for 90 min until the reaction was complete, as indicated by TLC analysis. The THF was evaporated under reduced pressure and the resulting residue was suspended in EDTA solution (10.0 mL). The water phase was extracted with $CH_2Cl_2$ (10.0 mL×3). The combined organic layers were dried over $Na_2SO_4$, filtered and concentrated. The resulting crude product was purified by column chromatography on silica gel (pure EtOAc) to yield **bola2E** as a colorless oil (262 mg, 65%).

$^1$H NMR (400 MHz, $CDCl_3$) δ 7.42 (s, 2H, C*H*), 6.98 (dd, *J* = 8.1, 2.2 Hz, 2H, Ar*H*), 6.70 (tt, *J* = 9.0, 2.4 Hz, 1H, Ar*H*), 4.77 (s, 1H, C*H*), 4.31 (t, *J* = 7.3 Hz, 4H, C$H_2$), 3.78 (s, 4H, C$H_2$), 3.65 (s, 12H, C$H_3$), 2.79 (t, *J* = 7.0 Hz, 8H, C$H_2$), 2.60 – 2.42 (m, 12H, C$H_2$), 1.94 – 1.81 (m, 4H, C$H_2$), 1.55 – 1.46 (m, 4H, C$H_2$), 1.37 – 1.15 (m, 28H, C$H_2$). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.92, 164.20, 164.07, 161.72, 161.59, 144.96, 144.73, 122.39, 110.91, 110.84, 110.72, 110.65, 103.53, 103.28, 103.03, 52.52, 51.61, 50.35, 49.00, 48.75, 32.69, 32.38, 30.36, 29.46, 29.44, 29.39, 29.16, 29.07, 29.04, 28.85, 26.53. $^{19}$F NMR (376 MHz, $CDCl_3$) δ -109.21. ESI-HRMS: calcd. for $C_{51}H_{82}N_8O_8S_2F_2$ $[M+2H]^{2+}$ 519.2905, found 519.2906.

**Synthesis of bola2A**

To a solution of **bola2E** (94 mg, 0.091 mmol) in methanol (2.0 mL) was added ethylenediamine (4.0 mL, 60 mmol). The reaction mixture was stirred for 72 h at 30 °C under argon. When the reaction was completed indicated by IR analysis, the reaction solution was evaporated, the obtained residue was purified by dialysis using dialysis tube of MWCO 1000, followed by lyophilization. After repeating 4 times the operation of dialysis and lyophilization, the product was lyophilized to give **bola2A** (99 mg, 95%) as a light-yellow oil.

$^1$H NMR (400 MHz, $CDCl_3$) δ 7.54 (br, 4H, N*H*), 7.48 (s, 2H, C*H*), 6.98 (dd, *J* = 8.0, 2.0 Hz, 2H, Ar*H*), 6.70 (tt, *J* = 8.8, 2.3 Hz, 1H, Ar*H*), 4.77 (s, 1H, C*H*), 4.30 (t, *J* = 7.3 Hz, 4H, C$H_2$), 3.75 (s, 4H, C$H_2$), 3.27 (q, *J* = 5.7 Hz, 8H, C$H_2$), 2.89 – 2.65 (m, 16H, C$H_2$), 2.61 – 2.36 (m, 12H, C$H_2$), 1.92 – 1.83 (m, 4H, C$H_2$), 1.58 – 1.46 (m, 4H, C$H_2$), 1.38 –

1.20 (m, 28H, C*H*$_2$). $^{13}$C NMR (101 MHz, $CDCl_3/CD_3OD$) δ 173.52, 168.64, 163.97, 163.42, 143.01, 138.22, 123.24, 120.94, 110.74, 110.48, 107.22, 103.09, 96.22, 50.43, 49.36, 47.33, 41.64, 40.76, 33.55, 32.26, 30.13, 29.32, 29.28, 29.00, 28.91, 28.68, 26.39. $^{19}$F NMR (376 MHz, $CDCl_3$) δ -109.62. ESI-HRMS: calcd. for $C_{55}H_{98}F_2N_{16}O_4S_2$ $[M+2H]^{2+}$ 575.3756, found 575.3754. HPLC (RT = 25.8 min)

**Synthesis of bola4E**

To the azide precursor **core** (103 mg, 0.18 mmol) were added $CuSO_4 \cdot 5H_2O$ (4.5 mg, 0.018 mmol), sodium ascorbate (7.2 mg, 0.036 mmol) and a solution of **4E** (280 mg,0.44 mmol) in 5.0 mL THF. The vessel was sealed and purged with argon for 5 min and 1.3 mL $H_2O$ was then added into the mixture. The reaction mixture was stirred at 50 °C for 90 min until the reaction was complete, as indicated by TLC analysis. The THF was evaporated under reduced pressure and the resulting residue was suspended in EDTA solution (10.0 mL). The water phase was extracted with $CH_2Cl_2$ (10.0 mL×3). The combined organic layers were dried over $Na_2SO_4$, filtered and concentrated. The resulting crude product was purified by column chromatography on silica gel (EtOAc/MeOH = 3/1-2/1) to yield **bola4E** as a colorless oil (230 mg, 70%).

$^1$H NMR (400 MHz, $CDCl_3$) δ 7.53 (s, 2H, C*H*), 7.14 (t, *J* = 5.2 Hz, 4H, N*H*), 7.00 (dd, *J* = 8.1, 2.1 Hz, 2H, Ar*H*), 6.71 (tt, *J* = 8.8, 2.3 Hz, 1H, Ar*H*), 4.78 (s, 1H, C*H*$_2$), 4.31 (t, *J* = 7.4 Hz, 4H, C*H*$_2$), 3.84 (s, 4H, C*H*$_2$), 3.67 (s, 24H, C*H*$_3$), 3.29 (q, *J* = 5.7 Hz, 8H, C*H*$_2$), 2.90 – 2.66 (m, 24H, C*H*$_2$), 2.63 – 2.49 (m, 12H, C*H*$_2$), 2.49 – 2.37 (m, 24H, C*H*$_2$), 1.96 – 1.82 (m, 4H, C*H*$_2$), 1.59 – 1.47 (m, 4H, C*H*$_2$), 1.39 – 1.17 (m, 28H, C*H*$_2$). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 173.09, 172.16, 164.17, 164.04, 161.69, 161.56, 144.95, 143.77, 122.62, 114.39, 110.88, 110.63, 103.51, 103.25, 103.00, 100.27, 52.97, 52.50, 51.69, 50.30, 49.31, 47.71, 37.15, 33.68, 32.73, 32.37, 30.36, 29.71, 29.45, 29.42, 29.15, 29.04, 28.83, 26.55. $^{19}$F NMR (376 MHz, $CDCl_3$) δ -109.23. ESI-HRMS: calcd. for $C_{87}H_{146}N_{16}S_2O_{20}F_2$ $[M+4H]^{4+}$ 460.5157, found 460.5156.

**Synthesis of bola4A**

To a solution of **bola4E** (110 mg, 0.060 mmol) in methanol (5.0 mL) was added

ethylenediamine (8.5 mL, 127 mmol). The reaction mixture was stirred for 72 h at 30 °C under argon. When the reaction was completed indicated by IR analysis, the reaction solution was evaporated, the obtained residue was purified by dialysis using dialysis tube of MWCO 2000, followed by lyophilization. After repeating 4 times the operation of dialysis and lyophilization, the product was lyophilized to give **bola4A** (116 mg, 94%) as a white solid.

$^{1}$H NMR (500 MHz, $CDCl_3/CD_3OD$) δ 7.92 (s, 2H, C*H*), 7.11 (dt, *J* = 6.4, 3.2 Hz, 2H, Ar*H*), 6.86 (tt, *J* = 8.9, 2.3 Hz, 1H, Ar*H*), 4.98 (s, 1H, C*H*), 4.46 (t, *J* = 7.2 Hz, 4H, C$H_2$), 3.90 (s, 4H, C$H_2$), 3.34 (t, *J* = 6.1 Hz, 24H, C$H_2$), 2.93 – 2.76 (m, 40H, C$H_2$), 2.74 – 2.57 (m, 12H, C$H_2$), 2.57 – 2.36 (m, 24H, C$H_2$), 2.07 – 1.92 (m, 4H, C$H_2$), 1.73 – 1.57 (m, 4H, C$H_2$), 1.50 – 1.27 (m, 28H, C$H_2$). $^{13}$C NMR (125 MHz, $CDCl_3/CD_3OD$) δ 173.79, 173.22, 164.56, 164.16, 162.11, 161.89, 145.23, 143.31, 123.66, 110.65, 110.45, 102.77, 52.16, 50.20, 49.90, 49.02, 48.57, 48.40, 48.23, 48.06, 47.89, 47.72, 47.55, 47.17, 41.66, 40.76, 37.30, 33.58, 33.32, 32.13, 30.08, 29.22, 28.88, 28.82, 28.52, 26.29. $^{19}$F NMR (471 MHz, $CDCl_3/CD_3OD$) δ -110.49. ESI-HRMS: calcd. for $C_{95}H_{178}N_{32}S_2O_{12}F_2$ $[M+3H]^{3+}$ 516.6007, found 516.6005. HPLC (RT = 19.6 min).

**Synthesis of bola8E**

To the azide precursor **core** (34 mg, 0.058 mmol) were added $CuSO_4 \cdot 5H_2O$ (2.9 mg, 0.012 mmol), sodium ascorbate (6.0 mg, 0.029 mmol) and a solution of **8E** (250 mg,0.18 mmol) in 6 mL THF. The vessel was sealed and purged with argon for 5 min and 1.5 mL $H_2O$ was then added into the mixture. The reaction mixture was stirred at 50 °C for 3 h until the reaction was complete, as indicated by TLC analysis. The THF was evaporated under reduced pressure and the resulting residue was suspended in EDTA solution (10.0 mL). The water phase was extracted with $CH_2Cl_2$ (10.0 mL×3). The combined organic layers were dried over $Na_2SO_4$, filtered and concentrated. The resulting crude product was purified by MeOH/Ether precipitation for 3 times to yield **bola8E** as colorless oil (160 mg, 80%).

$^{1}$H NMR (500 MHz, $CDCl_3$) δ 7.74 (br, 4H, N*H*), 7.59 (s, 2H, C*H*), 7.06 (t, *J* = 4.9 Hz, 8H, N*H*), 7.03 – 6.96 (m, 2H, Ar*H*), 6.72 (tt, J = 8.7, 2.3 Hz, 1H, Ar*H*), 4.80 (s, 1H,

C*H*$_2$), 4.32 (t, *J* = 7.4 Hz, 4H, C*H*$_2$), 3.83 (s, 4H, C*H*$_2$), 3.67 (s, 48H, C*H*$_3$), 3.40 – 3.20 (m, 24H, C*H*$_2$), 2.98 – 2.68 (m, 56H, C*H*$_2$), 2.68 – 2.51 (m, 28H, C*H*$_2$), 2.51 – 2.29 (m, 56H, C*H*$_2$), 2.01 – 1.73 (m, 4H, C*H*$_2$), 1.60 – 1.45 (m, 4H, C*H*$_2$), 1.42 – 1.16 (m, 28H, C*H*$_2$). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 173.06, 172.32, 172.27, 163.90, 163.80, 161.92, 161.82, 144.94, 143.59, 122.77, 110.84, 110.63, 103.43, 103.23, 103.02, 52.96, 52.56, 51.65, 50.29, 49.93, 49.27, 47.45, 37.44, 37.20, 33.88, 33.61, 32.71, 32.37, 30.37, 29.43, 29.16, 29.05, 28.85, 26.58. $^{19}$F NMR (471 MHz, $CDCl_3$) δ -109.29. ESI-HRMS: calcd. for $C_{159}H_{274}N_{32}O_{44}S_2F_2$ $[M+7H]^{7+}$ 492.4314, found 492.4344.

**Synthesis of bola8A**

To a solution of **bola8E** (100 mg, 0.029 mmol) in methanol (5.0 mL) was added ethylenediamine (10 mL, 150 mmol). The reaction mixture was stirred for 72 h at 30 °C under argon. When the reaction was completed indicated by IR analysis, the reaction solution was evaporated, the obtained residue was purified by dialysis using dialysis tube of MWCO 2000, followed by lyophilization. After repeating 4 times the operation of dialysis and lyophilization, the product was lyophilized to give **bola8A** (91 mg, 81%) as a white solid.

$^1$H NMR (300 MHz, $CDCl_3$/$CD_3OD$) δ 7.83 (s, 2H, C*H*), 7.02 (d, *J* = 6.3 Hz, 2H, Ar*H*), 6.77 (tt, *J* = 9.1, 2.4 Hz, 1H, Ar*H*), 4.89 (s, 1H, C*H*), 4.36 (t, *J* = 7.1 Hz, 4H, C*H*$_2$), 3.80 (s, 4H, C*H*$_2$), 3.24 (t, *J* = 6.0 Hz, 56H, C*H*$_2$), 2.90 – 2.66 (m, 88H, C*H*$_2$), 2.66 – 2.48 (m, 28H, C*H*$_2$), 2.48 – 2.26 (m, 56H, C*H*$_2$), 1.96 – 1.83 (m, 4H, C*H*$_2$), 1.61 – 1.45 (m, 4H, C*H*$_2$), 1.44 – 1.13 (m, 28H, C*H*$_2$). $^{13}$C NMR (125 MHz, $CDCl_3$/$CD_3OD$) δ 173.86, 173.37, 173.19, 161.95, 161.85, 145.89, 143.39, 123.78, 110.61, 110.41, 102.78, 102.58, 52.15, 50.05, 49.80, 49.07, 48.27, 48.10, 47.93, 47.76, 47.59, 47.42, 47.25, 47.09, 41.17, 40.53, 37.27, 33.80, 33.46, 33.34, 32.03, 30.02, 29.19, 28.84, 28.77, 28.44, 26.22. $^{19}$F NMR (471 MHz, $CDCl_3$/$CD_3OD$) δ -111.02. ESI-HRMS: calcd. for $C_{175}H_{338}F_2N_{64}O_{28}S_2^{6+}$, $[M+6H]^{6+}$ 649.1148, found 649.1147. HPLC (RT = 15.3 min).

**Dynamic light scattering (DLS)**

For DNA/bola-dendrimer complexes, the DNA solution was mixed with certain

amount of dendrimer solution at N/P ratio of 2.0. The final amount of the DNA was 24 ng/μL. For siRNA/bola-dendrimer complex, the siRNA solution was mixed with certain amount of dendrimer solution at N/P ratio of 10. The final concentration of the siRNA was 1.0 μM. The sizes and ζ-potentials of the nucleic acids/dendrimer complexes solutions were measured using a NanoBrookOmni equipped with a standard 633 nm laser at 25 °C. The experiments were performed in triplicates.

**Gel retardation analysis of nucleic acids/dendrimer complexes**

The bola dendrimers were diluted to an appropriate concentration. The nucleic acids were 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 nucleic acids in each sample was 200 ng/well. The nucleic acids/bola-dendrimer complexes were analyzed by electrophoretic mobility-shift assays in 1% agarose gel in standard TAE buffer for 15 min. The nucleic acid bands were stained by GoodView nucleic acid dyes and detected by Automatic chemiluminescence imaging system (5200Muti) (Tanon, Shanghai, China).

**Stability of nucleic acids/dendrimer complexes**

For DNA, an aliquot of 1.4 μg of DNA and the indicated amounts of bola-dendrimer solutions at N/P ratio 1.0 were kept at 37 °C for 30 min. Afterwards, the DNA/bola-dendrimer complexes were incubated in the presence of DNase I (0.1 U/well) 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 2.0 μL of 25 U/mL heparin solution on ice. The mixtures were electrophoresed through a 1.2% agarose gel in standard TAE buffer. The DNA bands were stained using nucleic acid dyes and then detected using Automatic chemiluminescence imaging system (5200Muti) (Tanon). Naked DNA was served as a control.

For siRNA, an aliquot of 1.4 μg of siRNA and the indicated amounts of bola-dendrimer solutions at N/P ratio of 10 were kept at 37 °C for 30 min. Afterwards, the siRNA/bola-dendrimer complexes 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 release evaluation using heparin displacement 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 bola-dendrimer diluted in PBS at N/P ratio of 10 was incubated 10 min at 25 °C. Then the above bola-dendrimer 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, 2, 4, 8, 10, 20, 50 U/mL) diluted in PBS was added to the siRNA/bola-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.

**Molecular Dynamics simulations**

The complex of siRNA and DNA with bola4A and bola8A dendrimers, was achieved by adapting a consolidated procedure developed by our group [2-4]. Each complex was then solvated with a TIP3P[5] water molecules model, extending at least 20 Å from the solute. A suitable number of Na+ and Cl- counterions were added to neutralize the system and reach an ionic strength of 150 mM. Each complex molecular model was then subjected to extensive energy minimization. 20 ns molecular dynamics (MD) simulations at 298 K were then employed for system equilibration, and further, 100 ns MD were run for data production. The free energy of binding between the dendrimers and the siRNA was calculated according to a previously validated approach[2-4] based on the molecular

mechanics Poisson−Boltzmann methodology[6]. The identification of each dendrimer charges effectively involved in nucleic acid binding ($N_{eff}$), and the corresponding individual contribution afforded to the overall binding energy ($\Delta G_{bind,eff}$) was achieved by applying a per-residue free energy decomposition technique to each complex. All MD simulations and the relevant analyses were performed using the AMBER 20[7] suites of programs, running in parallel on our own CPU/GPU calculation cluster. Molecular graphics images were produced using the UCSF Chimera package[8].

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**Cell culture**

Human prostate cancer PC-3 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Beijing, China). Human embryonic kidney (HEK) 293 cells, human ovarian cancer SKOV-3 cells, human pancreatic cancer PANC-1 cells, human cervical cancer HeLa cell, HeLa cells stably expressing GFP (HeLa-GFP cells), mouse triple negative breast cancer luciferase-tagged 4T1 cells (4T1-luc cells), mouse fibroblast L929 cells and madin-darby canine kidney MDCK cells were purchased from Tongpai Biotechnology Co., Ltd. (Shanghai, China). Mouse melanoma luciferase-tagged B16-F10 cells (B16-F10-luc cells) were provided by Prof. Can Zhang (China Pharmaceutical University, Nanjing, China).

PC-3 cells were maintained in F12K (Gibco, California, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco). HEK293 cells were cultured in MEM (Hyclone, Utah, USA) supplemented with 10% FBS. SKOV-3 cells were maintained in McCOY'S 5A (Hyclone), supplemented with 10% FBS. PANC-1 cells, HeLa cells and HeLa-EGFP cells, were cultured in RPMI-1640 (Hyclone) supplemented with 10% FBS. 4T1-luc cells were cultured in RPMI-1640 (Hyclone) supplemented with 10% FBS and puromycin. L929 cells and MDCK cells were maintained in DMEM (Hyclone), supplemented with 10% FBS. B16-F10-luc cells were maintained in DMEM (Hyclone), supplemented with 10% FBS and puromycin. Cells were maintained at 37 °C with 5% $CO_2$ humidified atmosphere.

**PrestoBlue assay**

HEK 293 cells were seeded at $1.0 \times 10^4$ cells/well in 96-well plates. L929 and MDCK

cells were respectively seeded in 96-well plates ($4.0 \times 10^3$ cells/well). All the cells were allowed to grow overnight. The cells were then treated with various concentrations (0.01 - 100 μM) of bola-dendrimers for 48 h. Cell viability was estimated after addition of the CellTiter-Blue viability reagent (Promega) for 3 h according to the protocol provided by the supplier. Nontreatment was used as positive control. Cell viability was normalized with respect to non-treated cell rates. All samples were run in triplicate.

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

SKOV-3, PC-3, HeLa and PANC-1 cells were respectively seeded in 96-well plates ($5.0 \times 10^3$ cells/well) and allowed to attach overnight. The cells were then treated with bola-dendrimers and nucleic acids/bola-dendrimer complexes. 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). Nontreatment was set as positive control. 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.

**Lactate dehydrogenase (LDH) assay**

The different cell lines were seeded in 96-well plates and cultured overnight. The density of HEK293 cells was $1.0 \times 10^4$ cells per well. The density of MDCK cells and L929 cells was $4.0 \times 10^3$ cells per well. The density of SKOV-3, PC-3, HeLa and PANC-1 cells was $5.0 \times 10^3$ cells per well. Then, HEK293, MDCK and L929 cells were respectively treated with various concentrations (0.01 - 50 μM) of bola-dendrimers for 48 h. SKOV-3, PC-3, HeLa, PANC-1 cells were respectively treated with bola-dendrimer alone and nucleic acids/bola-dendrimer complexes for 8 h. Afterwards, cell membrane damage toxicity was determined using commercial LDH assay kit (Dojindo Laboratories, Shanghai,

China). 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 50.0 μL of stop solution. The optical density (OD) of these solutions were measured at 490 nm via Cytation5 Microplate Reader (BioTek). Lysis buffer and medium were used as positive control and negative control, 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%.

**Hemolysis experiment**

Red blood cells (RBCs) were isolated from 2.0 mL fresh blood collected from healthy Kunming mice by centrifuged at $5 \times 10^3$ 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 18 mL PBS, and 0.50 mL of such RBC suspension was added to 0.50 mL of suspension containing different concentrations of bola-dendrimer assemblies at 1.6, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 9.0, 10, 50, and 100 μM in PSB buffer to offer the final concentration of 0.8, 1.0, 1.25, 1.5, 2.0, 2.5, 3.0, 4.5, 5.0, 10, 25, 50 μM, respectively. 0.50 mL RBC suspensions incubated with 0.50 mL PBS and 0.50 mL 1.0% TritonX-100 solution (Beyotime Biotechnology, Shanghai, China) were used as negative and positive controls, respectively. The samples were mixed gently, left at room temperature for 2 h, and then centrifuged at $1.0 \times 10^4$ 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). Control was performed with 1.0% TritonX-100 solution and PBS buffer, and set as 100% and 0% hemolysis, respectively. The percentage of hemolysis was calculated as follows:

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

**ROS level measurement**

SKOV-3 and NAC-treated SKOV-3 cells were respectively seeded in 6-well plates ($3.0 \times 10^5$ cells/well) and incubated for 24 h. Then the cell pellets were collected by centrifugation and placed into 1.0 mL complete growth medium. The CellROX® orange Reagent (Thermo Fisher Scientific, Carlsbad, USA) was then added at a final concentration of 0.50 μM to the cells and incubated for 15 minutes at 37 °C in the dark. Afterwards, the cells were washed with PBS three times, mean fluorescence intensities from the cells were quantified by using Attune NxT flow cytometry (Thermo Fisher Scientific). Each assay was performed in triplicate.

***In vitro* transfection of nucleic acids/dendrimer complexes**

*DNA delivery*

One day before transfection, SKOV-3, HeLa, and PC-3 cells were respectively seeded in 24-well plates ($5.0 \times 10^4$ cells/well) in 0.50 mL of fresh complete medium containing 10% FBS. The DNA/bola-dendrimer complexes solution was prepared at N/P ratio of 1.0 before transfection. The desired amount of DNA and bola-dendrimer was diluted in 25 μL of Opti-MEM transfection medium. The solutions were mixed gently by pipette and then left 10 min at room temperature. The bola-dendrimer solution was added to the DNA solution, homogenized for 10 s and left 30 min at room temperature for the formation of DNA/bola-dendrimer complexes. Then 0.20 mL of Opti-MEM medium was added into the complexes solution and the final volume brought to 0.25 mL. Before addition of the transfection complexes, the complete medium with serum was removed and cells were washed with PBS once. Then, 0.25 mL of the complex's solution was added and incubated at 37 °C in the absence of 10% FBS. 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 48 h for detecting GFP fluorescence expression by fluorescence microscope (Zess, Jena, Germany) and Attune NxT flow cytometry (Thermo Fisher Scientific) or 72 h for western blot analysis. The commercial transfection reagent Lipofectamine 2000 (Lipo) (Thermo Fisher Scientific) was used as positive control.

*siRNA delivery*

*Transfection in HeLa-GFP cells:* HeLa-GFP cells were seeded in 24-well plates ($3.0 \times 10^5$ cells/well) in 500 μL of complete medium 24 h prior to transfection. A solution of the siRNA/bola-dendrimer complexes was prepared at N/P ratio of 10 before transfection. The desired amount of EGFP siRNA and bola-dendrimer was diluted in 25 μL of Opti-MEM transfection medium. The solutions were mixed by pipette and then left for 10 min at room temperature. The bola-dendrimer was added to the siRNA solution, homogenized for 10 s and then equilibrated 30 min at room temperature for the formation of siRNA/bola-dendrimer complexes. Then 0.20 mL of Opti-MEM medium was added into the complexes solution and the final volume brought to 0.25 mL. Before adding the transfection complexes, the complete medium with serum was removed and cells were washed by PBS once. Then, 0.25 mL of the complexes solution was added and incubated at 37 °C in absence of 10% FBS. 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. The commercial transfection reagent Lipofectamine 2000 (Lipo) (Thermo Fisher Scientific) was used as positive control. GFP protein expression was determined by fluorescence microscope (Zess) and Attune NxT flow cytometry (Thermo Fisher Scientific).

*Transfection in other cancer cell lines:* PC-3, SKOV-3, and PANC-1 cells were seeded in 6-well plates ($8.0 \times 10^4$ cells/well) and cultured in 2.0 mL of fresh medium containing 10% FBS one day before transfection. The siRNA/bola-dendrimer complexes solution was prepared at N/P ratio of 10 before transfection. The desired amount of siRNA and bola-dendrimer 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 bola-dendrimer 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 complexes solution and the final volume brought to 1.0 mL. Before addition of the transfection complexes, the complete medium with serum was removed and cells were washed by PBS once. Then, 1.0 mL of the solution of the transfection complexes 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 dioleoylphosphatidylethanolamine (DOPE)*

The transfection experiments involving DOPE were performed in SKOV-3 cells as described above, except the desired amount of bola-dendrimer 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 SKOV-3 cells were pre-incubated with 200 nM bafilomycin A1 at 37 ºC for 1 h.

**Cell uptake of nucleic acids/bola-dendrimer complexes**

*Flow cytometry.*

SKOV-3 cells were seeded at $5.0 \times 10^4$ cell/well in a 24-well plate one day before transfection. The cells were incubated with YOYO-1 labeled DNA/bola-dendrimer complexes (12 ng/μL YOYO-1 labeled DNA, N/P ratio 1.0) or Cy5 labeled siRNA/bola-dendrimer complexes (50 nM Cy5-siRNA, N/P ratio 10) for 15, 30, 60 120 and 240 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 ($8.0 \times 10^4$ cells/dish) one day before transfection. The cells were incubated with YOYO-1 labeled DNA/bola-dendrimer complexes (12 ng/μL YOYO-1 labeled DNA, N/P ratio 1.0) or Cy5 labeled siRNA/bola-dendrimer complexes (50 nM Cy5-siRNA, N/P ratio 10) for 4 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).

**Western blot analysis**

Samples containing equal amounts of proteins from cultured cells (HeLa, PC-3, SKOV-3 or PANC-1 cells) or tumors (SKOV-3 or HeLa xenograft tumors) 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 survivin rabbit polyclonal antibody (Cell signaling Technology, Massachusetts, USA, 1:1000), anti-human p53 rabbit polyclonal antibody (Cell signaling Technology, Massachusetts, USA, 1:1000), anti-human pTRAIL rabbit polyclonal antibody (Cell signaling Technology, Massachusetts, USA, 1:1000) or anti-human HSP27 rabbit polyclonal antibody (Enzo Life Sciences, Villeurbanne, France, 1:50000), anti-human vinculin rabbit polyclonal antibody (Sigma-Aldrich, Shanghai, China, 1:10000) or anti-mouse GAPDH rabbit polyclonal antibody (Santa Cruz Biotechnology, Beijing, China, 1:1000) or anti-human AKT2 rabbit polyclonal antibody (Cell signaling Technology, Massachusetts, USA, 1:1000) at 4 °C overnight. Then, after washing, the membranes were incubated with anti-rabbit or anti-mouse monoclonal secondary antibodies (Invitrogen, Massachusetts, USA, 1:5000) for 2 h at 25 °C. Specific proteins were detected using an enhanced-chemiluminescence Western blotting analysis system (Tanon).

**Quantitative real-time (qRT)-PCR analysis**

The total RNA of SKOV-3 tumors was isolated with the Trizol method (Vazyme Biotech Co. Ltd, Nanjing, China) and reverse-transcribed using a Reverse Transcription Kit (Vazyme Biotech Co. Ltd, Nanjing, China). Expression of the target genes was analyzed by quantitative Real time-PCR performing on the QuantStudio3™ Real-Time PCR System (Applied Bisystems, Thermo Fisher Scientific) using 2×SYBR Green (Vazyme Biotech Co. Ltd, Nanjing, China). β2M expression was used for normalization of the qPCR data. Primers were used as follows: AKT2 forward primer: 5'-CAACGGGGGTGAGCTGTT-3'; AKT2 reverse primer: 5'-CGCACATCATCTCG TACATGACC-3'; β2M forward primer: 5’-GAGGCTATCCAGCGTACTCCAA-3’; β2M reverse primer: 5’-AGTCAACTTCAATGTCGGATGGA-3’ (GenScript Biotech Corp., Nanjing, China).

**Biodistribution of nucleic acids/bola dendrimers in mice.**

All animals involved in this work were maintained in China Pharmaceutical University Laboratory Animal Center. All procedures were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University and performed in accordance with the guidelines and policies. The approval number is "2021-10-013". Nude mice bearing SKOV-3 xenografts were intravenously injected with Cy5 labeled siRNA/**bola4A** complex (1.0 mg/kg, 3.9 mg/kg **bola4A**, N/P ratio of 5.0), Cy5 labeled siRNA alone (1.0 mg/kg), Cy5 labeled DNA/**bola8A** complex (0.25 mg/kg, 0.2 mg/kg **bola8A**, N/P ratio of 1.0), Cy5 labeled DNA alone (0.25 mg/kg) or PBS buffer. 24 h post administration, mice were sacrificed by cervical dislocation, and the major organs as well as the tumors were isolated for ex vivo imaging (PerkinElmer IVIS Spectrum CT, Massachusetts, USA). Finally, quantitative analysis was performed using a software package included with the imaging system.

**Toxicity assessment in healthy mice**

All animals involved in this work were maintained in China Pharmaceutical University Laboratory Animal Center. All procedures were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University and performed in accordance with the guidelines and policies. The approval number is "2021-12-021". 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 injected with, respectively, PBS buffer, **bola8A** alone, DNA/**bola8A** (1.0 mg/kg DNA, 0.7 mg/kg **bola8A**, N/P ratio of 1.0), **bola4A** alone, and scramble siRNA/**bola4A** (3.0 mg/kg siRNA, 11.6 mg/kg **bola4A**, N/P ratio of 5.0). After 24 h, 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.

### *In vivo* DNA delivery in cervical cancer HeLa xenograft mouse model

All animals involved in this work were maintained in China Pharmaceutical University Laboratory Animal Center. All procedures were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University and performed in accordance with the guidelines and policies. The approval number is "2021-10-012". HeLa cells were inoculated subcutaneously in the flank region of 5-week-old female BALB/c nude mice. When HeLa tumors reached 50 $mm^3$, mice were randomly divided into 3 groups (3 mice per group), and were then intravenously treated with **bola8A** alone, p53/**bola8A** (1.0 mg/kg DNA, 0.7 mg/kg **bola8A**, N/P ratio 1.0), p53 (1.0 mg/kg) and PBS buffer, respectively, twice per week. The size of the tumors and the weight of mice were recorded once per week. All the mice were sacrificed after 3 weeks of treatments. The tumors were collected and the weight of tumors were recorded. The tumors were divided into two parts. One part of the tumors was frozen in liquid nitrogen and then stocked at -80 °C for protein extraction. The *in vivo* expression of p53 protein was measured by Western blot assay as described above. Another part of tumors was fixed in 4% paraformaldehyde for TUNEL analysis to access the cell apoptosis and immunohistochemistry analysis using Ki67 staining to evaluate cell proliferation *in vivo*. The serum specimens were collected to measure biochemical parameters. Tissue specimens of different organs (hearts, lungs, livers, kidneys, and spleens) were fixed in 10% neutral-buffered formalin and embedded in paraffin for histopathology analyses including H&E staining, Ki67 staining, and TUNEL assay.

### *In vivo* siRNA delivery in ovarian cancer SKOV-3 xenograft mouse model

All animals involved in this work were maintained in China Pharmaceutical University Laboratory Animal Center. All procedures were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University and performed in accordance with the guidelines and policies. The approval number is "2021-10-012". SKOV-3 cells were inoculated subcutaneously in the flank region of 5-week-old female BALB/c nude mice. When SKOV-3 tumors reached 50 $mm^3$, mice were randomly divided

into 5 groups (3 mice per group), and were then intravenously treated with, respectively, **bola4A** alone, siAKT2/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio 5.0), scramble siRNA/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio 5.0), siAKT2 alone (1.0 mg/kg siRNA) and PBS buffer twice per week. All the mice were sacrificed after 3 weeks of treatments. The tumors were collected and the weight of tumors were recorded. The tumors were divided into two parts. One part of the tumors was frozen in liquid nitrogen and then stocked at -80 °C for RNA and protein extraction. The expression of AKT2 mRNA was measured by qRT-PCR analysis as described above. The expression of AKT2 protein was measured by Western blot assay as described above. Another part of tumors was fixed in 4% paraformaldehyde for TUNEL analysis to access the cell apoptosis and immunohistochemistry analysis using Ki67 staining to evaluate cell proliferation *in vivo*. The serum specimens were collected to measure biochemical parameters. Tissue specimens of different organs (hearts, lungs, livers, kidneys, and spleens) were fixed in 10% neutral-buffered formalin and embedded in paraffin for histopathology analyses including H&E staining, Ki67 staining, and TUNEL assay.

***In vivo* DNA and siRNA delivery in metastatic breast cancer model**

All animals involved in this work were maintained in China Pharmaceutical University Laboratory Animal Center. All procedures were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University and performed in accordance with the guidelines and policies. The approval number is “2022-07-006”. Luciferase-tagged metastatic murine triple negative 4T1 (4T1-luc) cells were injected into the tail vein of BALB/c female mice (10 weeks old, Shanghai Xipur-Bikai Experimental Animal Ltd, Shanghai, China). One day post tumor challenge, mice were randomly divided into 5 groups (5 mice per group), and were then intravenously treated with, respectively, PBS buffer, p53 (2.0 mg/kg), p53/**bola8A** (2.0 mg/kg DNA, 1.5 mg/kg **bola8A**, N/P ratio 1.0), p53/Lipo complex (2.0 mg/kg), p53/JetPEI (2.0 mg/kg DNA, N/P ratio 1.0), scramble siRNA/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio 5.0), siAKT2/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio 5.0), and siAKT2/MC3 (1.0 mg/kg siRNA) every 2 days for six injections in total. The tumor metastases were detected and quantified

in vivo in the 4T1-luc metastatic tumor-bearing mice and ex vivo in the lung of the mice by bioluminescence imaging (PerkinElmer IVIS Spectrum CT, Massachusetts, USA). Briefly, for luciferase-expressing tumors, mice were injected i.p. with 150 mg/kg D-luciferin (YEASEN CO., Ltd, Shanghai, China) and photon flux was measured about 15 min later in vivo as well as ex vivo dissected lungs. The lungs were excised, fixed in 4% polyformaldehyde for 24 h and embedded in paraffin. Then histopathology analysis was performed using H&E staining to access the tumor cell metastasis and immunohistochemistry to evaluate protein expression of p53 or AKT2 in tumor. The metastasis lesions in histological images of lungs were quantified by using Image J software.[9] The commercial gene transfection reagent Lipofectamine 2000 (Lipo) and in vivo-JetPEI (Polyplus-transfection, Vectura, France) were used as positive controls for in vivo DNA delivery. Dlin-MC3-DMA (MC3) (AVT Pharmaceutical Tech Co., Ltd., Shanghai, China), an FDA-approved LNP for siRNA delivery, was used as positive control for in vivo siRNA delivery.

***In vivo* DNA and siRNA delivery in metastatic melanoma model**

All animals involved in this work were maintained in China Pharmaceutical University Laboratory Animal Center. All procedures were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University and performed in accordance with the guidelines and policies. The approval number is "2022-07-006". Luciferase-tagged metastatic melanoma B16-F10 (B16-F10-luc) cells were injected into the tail vein of C57BL6 female mice (6 weeks old, Shanghai Xipur-Bikai Experimental Animal Ltd, Shanghai, China). One day post tumor challenge, mice were randomly divided into 5 groups (5 mice per group). For DNA delivery, mice were then intravenously administrated with, respectively, PBS buffer, p53 (2.0 mg/kg), p53/**bola8A** (2.0 mg/kg DNA, 1.5 mg/kg **bola8A**, N/P ratio 1.0), and p53/JetPEI (2.0 mg/kg DNA, N/P ratio 1.0) every 2 days for six injections in total. For siRNA delivery, mice were then intravenously treated with, respectively, PBS buffer, scramble siRNA/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio 5.0), siAKT2/**bola4A** (1.0 mg/kg siRNA, 3.9 mg/kg **bola4A**, N/P ratio 5.0), and siAKT2/MC3 (1.0 mg/kg siRNA) every 2 days for four injections in total.

The tumor metastases were detected and quantified in the lung of the mice using bioluminescence imaging (PerkinElmer IVIS Spectrum CT). Briefly, for luciferase-expressing tumors, mice were injected i.p. with 150 mg/kg D-luciferin (YEASEN CO., Ltd) and photon flux was measured about 15 min later in vivo as well as ex vivo dissected lungs. The lungs were excised, fixed in 4% polyformaldehyde for 24 h and embedded in paraffin. Then histopathology analysis was performed using H&E staining to assess the tumor cell metastasis and immunohistochemistry to evaluate protein expression of p53 or AKT2 in tumor. The metastasis lesions in histological images of lungs were quantified by using Image J software.[9] The commercial gene transfection reagent in vivo-JetPEI was used as positive control for in vivo DNA delivery. Dlin-MC3-DMA (MC3), an FDA-approved LNP for siRNA delivery, was used as positive control for in vivo siRNA delivery.

**Statistical tests**

All data are presented as mean ± SD unless otherwise indicated. Statistical analysis was performed by one-way analysis of variance (ANOVA), two-way ANOVA, unpaired Student's t-test or Mann-Whitney test (GraphPad Prism 8.01). A p value $<0.05$ was considered statistically significant, whereby all significant values in various figures are indicated as follows: $^{*}p\leq0.05$; $^{**}p\leq0.01$; $^{***}p\leq0.001$.

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