Electronic Supplementary Information

A DR4 capturer with AKT siRNA for synergetic enhancement of death receptor-mediated apoptosis

Wei Pan,‡ Shaohua Gong,‡ Yanhua Li, Huiwen Zhang, Na Li and Bo Tang**

College of Chemistry, Chemical Engineering and Materials Science, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Institute of Molecular and Nano Science, Shandong Normal University, Jinan 250014, P. R. China.

*E-mail: lina@sdnu.edu.cn; tangb@sdnu.edu.cn

Experimental details:

Materials. carboxyl-functionalized graphene oxide (GO) was purchased from Nanjing XFNANO Materials Tech Co., Ltd (Nanjing, China). N-Hydroxysulfosuccinimide (Sulfo-NHS), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), 1-pyrenemethylamine hydrochloride ($PyNH_2$) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Shanghai, China). Anti-DR4 antibody, anti-FADD antibody, anti-caspase-8 antibody, anti-caspase-3 antibody and secondary antibody (Alexa Fluor 647) were purchased from Abcam (Shanghai, China). Cell culture products, unless mentioned otherwise, were purchased from GIBCO. The cervical cancer cell line (HeLa), the Human hepatocellular liver carcinoma cell line (HepG2) and the human hepatocyte cell line (HL-7702) were obtained from the Committee on Type Culture Collection of the Chinese Academy of

Sciences. The human breast cancer cell line MCF-7 was acquired from KeyGen Biotech. Co. Ltd. (Nanjing, China).

Instruments. Transmission electron microscopy (TEM) was carried out on a JEM-100CX II electron microscope. UV-Vis absorption spectra were measured on pharmaspec UV-1700 UV-Visible spectrophotometer (Shimadzu, Japan). RT-PCR was carried out with LineGene 9620 sequence detection system (Bioer, Hangzhou, China). Flow cytometry was performed using a Beckman Coulter Epics XL (Beckman Coulter, Inc., Brea, CA). Nanodrop experiment was performed on NanoDrop Micro-UV/Vis Spectrophotometers (Thermo Fisher Scientific Inc, USA). MTT assay was measured in a microplate reader (Synergy 2, Biotek, USA). Dynamic light scattering and zeta potential measurements were performed with Malvern Zeta Sizer Nano (Malvern Instruments). Confocal fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with an objective lens (×20).

Synthesis of oligonucleotides. RNA oligonucleotides were synthesized and HPLC purified by TAKARA Biotechnology Co., Ltd (Dalian, China). The sequence of the RNA oligonucleotides was listed in Table S1.

Preparation of the nanocarrier. 5 mM Sulfo-NHS and 1 mM EDC were added into the GO (1 mg/mL pH6.0), then the mixture was bath sonicated for 30 min. After that the pH of the mixture was adjusted to 8.0 with 1 mM NaOH, then anti-DR4 antibody (6 μg/mL) was added. The mixture was shaken overnight at room temperature. Then the mixture was centrifuged at 14,000 rpm for 30 min and resuspended in PBS. After

that $PyNH_2$(0.1 mg/mL) was added and sonication bath at room temperature for 24 h, then 200 nM AKT siRNA was added into the mixture and kept at room temperature for 2 h. The product was purified by repeated centrifugation at 14, 000 rpm for 30 min and resuspended in PBS.

Nanodrop experiment. AKT siRNA (200 nM) was added to 1 mg/mL GO, and the mixture was incubated for 2 h at room temperature. After this, centrifugation was carried out and a supernate was obtained. NanoDrop Micro-UV/Vis Spectrophotometers was used to obtain the concentration of the AKT siRNA in the supernate. Total AKT siRNA without reaction with GO was also measured. Then the percentage of the AKT siRNA on nanocarrier was calculated by: (total AKT siRNA concentration -AKT siRNA concentration in supernate) /total AKT siRNA concentration *100%.

DR4 expression and aggregation. HepG2 and HL-7702 cells were used in this experiment. Both of the cells were divided into two groups. One group was incubated with culture medium containing DR4 antibody for 12 h. The other group were incubated with nanocarrier for 12 h. After washing with PBS and fixing by 4% paraformaldehyde in PBS for 10 min, the cells were washed with PBS. Afterwards, the cells were incubated with primary antibody (DR4 antibody) for 1 h and washed with PBS thrice (5 min each). The cells were then incubated with secondary antibody (Alexa647-labeled antibody) for 45 min and washed with PBS thrice, followed by staining with Hochst33342 at 37 °C for 10 min. Subsequently, the cells were washed with ice-cold PBS twice and immediately observed using CLSM.

Flow cytometry. HepG2 and HL-7702 cells were used in this experiment. Both of the cells were incubated with nanocarrier for 12 h. The cells were detached from culture flasks using trypsin. After washing with culture medium and PBS, then the cells were fixed by 4% paraformaldehyde in PBS for 10 min, after that the cells were washed with PBS. Afterwards, the cells were incubated with primary antibody (FADD antibody) for 1 h and washed with PBS thrice (5 min each). The cells were then incubated with secondary antibody (Alexa647-labeled antibody) for 45 min and washed with PBS thrice. Subsequently, the cells were washed with ice-cold PBS twice and flow cytometry was performed using a Beckman Coulter Epics XL, with excitation at 647 nm.

Gene silencing effect. GO loaded with AKT siRNA was used to evaluate the gene silencing effect of AKT siRNA. HepG2 cells were incubated with GO loaded with AKT siRNA (100 μg/mL) for 12 h, then RT-PCR was carried out to determine the expression of AKT mRNA.

Immunofluorescence staining of DR4, FADD, Caspase-8 and Caspase-3. HepG2 and HL-7702 cells were used in this experiment. Both of the cells were incubated with nanocarrier for 12 h. After washing with PBS and fixing by 4% paraformaldehyde in PBS for 10 min, the cells were washed with PBS. Afterwards, the cells were incubated with primary antibody (DR4 antibody, FADD, Caspase-8 or Caspase-3 antibody, respectively) for 1 h and washed with PBS thrice (5 min each). The cells were then incubated with secondary antibody (Alexa647-labeled antibody) for 45 min and washed with PBS thrice, followed by staining with Hoechst 33342 at 37 °C for 10 min.

Subsequently, the cells were washed with ice-cold PBS twice and immediately observed using CLSM.

MTT assay. MCF-7, HepG2, HeLa and HL-7702 cells were used in this experiment. All of the cells were seeded in 96-well plates and incubated at 37 °C in 5% CO_2 for 24 h. Then all of the cells were divided into four groups in parallel. Group 1 was incubated with culture medium and set as control; group 2 was incubated with culture medium contained GO (100 μg/mL); group 3 was incubated with culture medium contained GO loaded with AKT siRNA only; group 4 was incubated with culture medium contained GO loaded with DR4 antibody only; group 5 was incubated with culture medium contained GO loaded with DR4 antibody and AKT siRNA. After 12 h incubation all of the culture medium was replace with fresh medium and incubated for 2 days. Next, 150 μL MTT solution (0.5 mg/mL) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a microplate reader.

***In vivo* experiment.** All animal experiments were carried out according to the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China. Female nude mice (4-6 weeks old, ~20 g) were housed under normal conditions with 12 h light and dark cycles and given access to food and water ad libitum. MCF-7 cells were used in this experiment. Nude mice were subcutaneously injected with 200 μL MCF-7 cells (5×10^6 cells/mL) and divided into four group. Four days after injection, group B was intratumor injected with GO loading with siRNA, group C was

intratumor injected with GO loading with DR4 antibody and group D was intratumor injected with GO loaded with AKT siRNA and DR4 antibody every two days. The tumor size was monitored every two days and tumors were harvested after 10 days. The morphology and weight of the tumors were recorded.

Supplementary Table:

Table S1： The sequence of the RNA oligonucleotides

Oligonucleotide	Sequence
AKT siRNA	5’-AAGGAGCGGCCGCAGGAUGUGAAAAAA-$(CH_2)_3$-SH-3’ 3’-UUCCUCGCCGGCGUCCUACAC-5’
AKT forward primer	5’-TCTATGGCGCTGAGATTGTG-3’
AKT reverse primer	5’-CTTAATGTGCCCGTCCTTGT-3’
GAPDH forward primer	5’-GGGAAACTGTGGCGTGAT-3’
GAPDH reverse primer	5’-GAGTGGGTGTCGCTGTTGA-3’

Supplementary Figures:

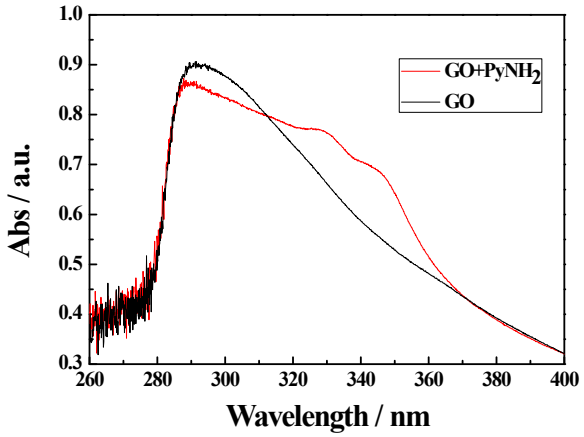

Figure S1. The UV-Vis absorption spectra of GO and GO loaded with $PyNH_2$ (GO+$PyNH_2$).

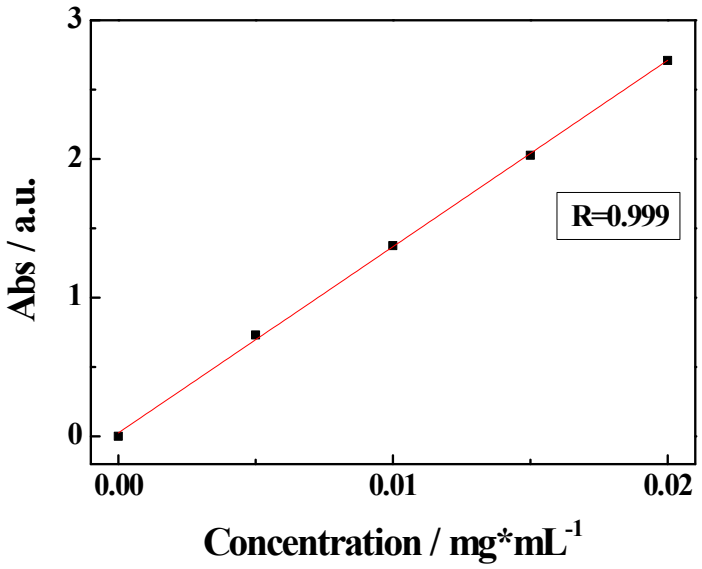

Figure S2. The absorbance standard curve line of $PyNH_2$.

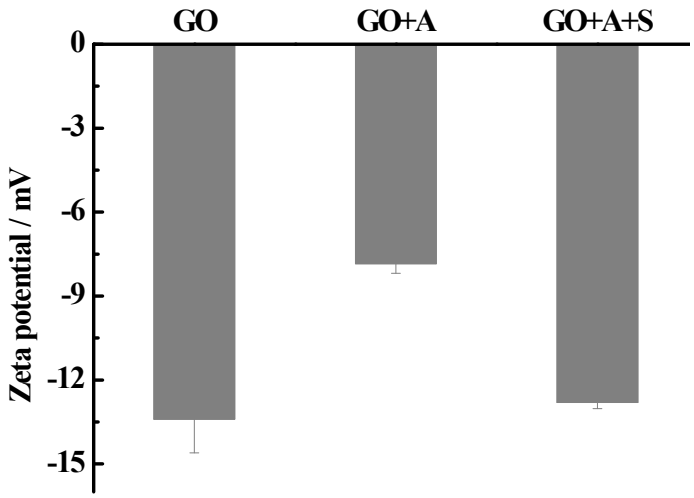

Figure S3. Zeta potential of GO, GO loaded with DR4 antibody（GO+A）and GO loaded with DR4 antibody and AKT siRNA (GO+A+S).

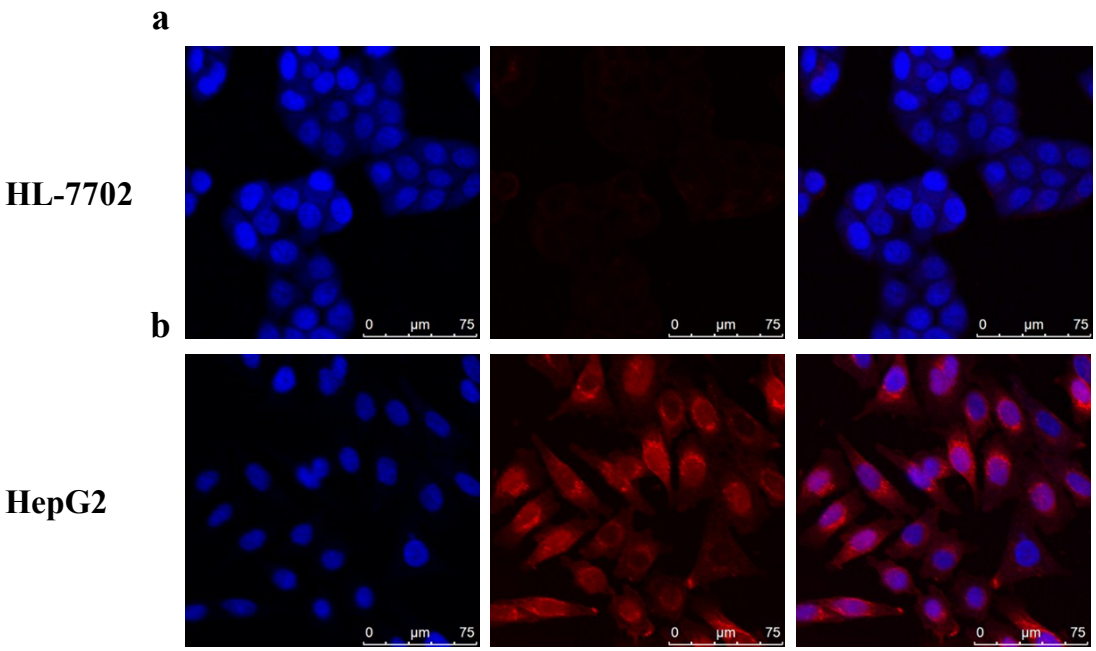

Figure S4. The expression of DR4s on the surface of (a) HL-7702 and (b) HepG2 cells.

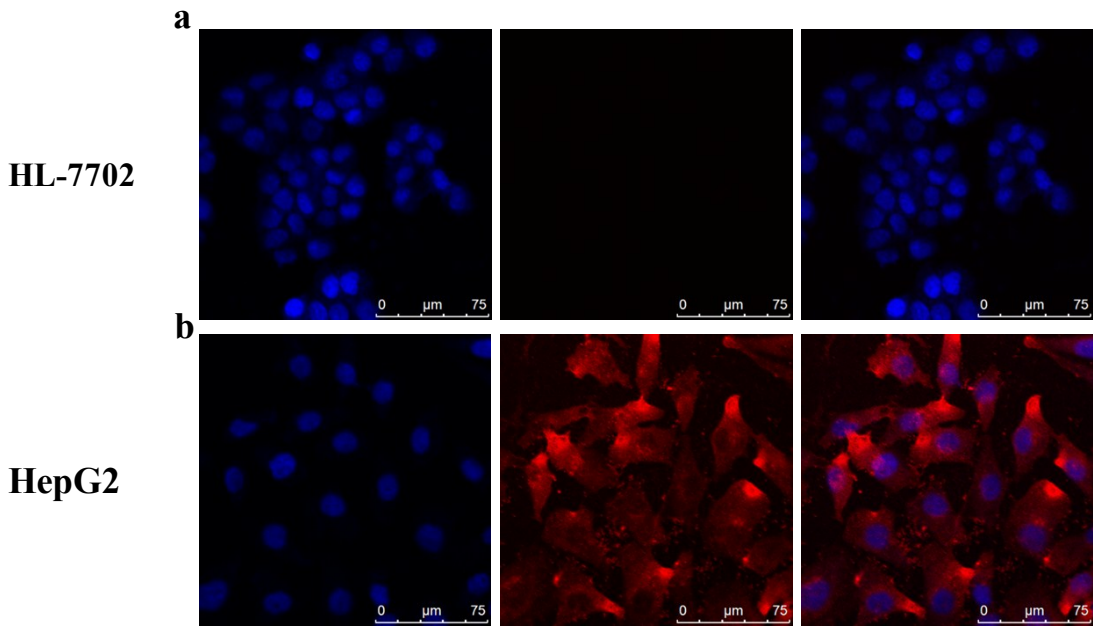

Figure S5. Clustering of DR4s induced by nanocarrier. (a) HL-7702 and (b) HepG2 cells.

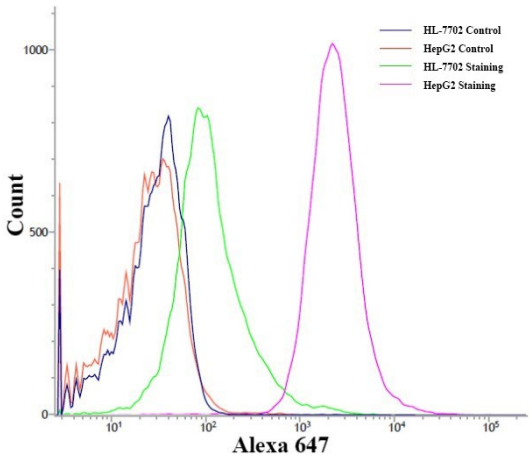

Figure S6. Flow cytometry data of the receptor clustering. HepG2 and HL-7702 cells were incubated with nanocarrier and staining with FADD antibody.

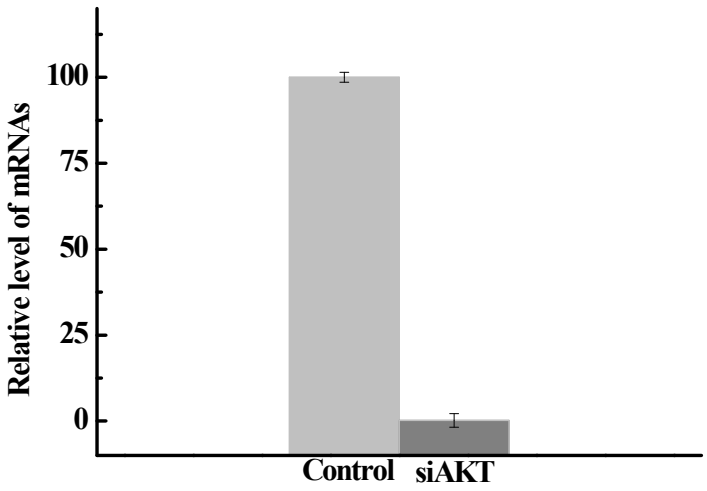

Figure S7. Gene silencing effect of GO loaded with AKT siRNA.