Supplementary Information

Spatial Confinement of Chemically Engineered Cancer Cells Using Large Graphene Oxide Sheets: a New Mode of Cancer Therapy

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Fig. S14 H&E staining images of some organs and the subcutaneous tumors collected from the mice with different treatments. $n = 4$. Original magnification: 20 ×.

Fig. S15 Tumor growth curves (tumor mass volume) of the subcutaneous tumors after different treatments. $n = 4$. p values were calculated by the Student's t-test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table S1 Sequences of oligonucleotides.

Table S2 Primer sequences used for qRT-PCR.

Raw data

Experimental Section

Chemicals and materials

All DNA oligonucleotides were synthesized by Takara Bio Inc. (Shiga Prefecture, Japan) and purified by high-performance liquid chromatography (HPLC). The sequences are shown in Table S1 and S2. Tetra-acylated N-azidoacetylmannosamine (Ac_4ManNAz) was obtained from Click Chemistry Tools LLC (Scottsdale, USA). Dulbecco's phosphate-buffered saline (DPBS), streptavidin-horseradish peroxidase (HRP), crystal violet and epidermal growth factor (EGF) were purchased from Sangon Biotechnology Inc. (Shanghai, China). Graphene oxide (GO) materials were obtained from Nanjing XFNANO Materials Tech Co., Ltd. (Nanjing, China). Green fluorescent protein (GFP) was purchased from Beyotime (Shanghai, China). The primary antibody of c-Fos, bRaf, phospho-bRaf, cRaf, phospho-cRaf, MEK and phospho-MEK were obtained from Cell Signaling Technology Inc (Danvers, MA, USA). Phalloidin-iFluor 488, other primary antibodies and all second antibodies used for Western Blotting and immunofluorescence were obtained from Abcam (Cambridge, MA, USA). Dil, 4', 6-diamidino-2-phenylindole (DAPI), Cell counting kit-8 assay (CCK-8), PI/Annexin V-FITC apoptosis detection kit and H&E staining solution were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Dibenzocyclooctyne (DBCO)-biotin, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxy succinimidepurum (NHS) and other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). All solutions were prepared with Milli-Q water (18.2 MΩ cm^{-1}) from a Milli-Q purification system (Millipore, Milford, MA, USA).

Cell culture

Human non-small-cell lung cancer A549 cells, human breast cancer MDA-MB-231 cells and human cervical cancer HeLa cells were obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Science). A549 cells and HeLa cells were cultured in DMEM-high glucose medium (KeyGEN, Nanjing, China) supplemented with 10% FBS (Gibco, Invitrogen), 1% penicillin and streptomycin. MDA-MB-231 cells were maintained in RPMI1640 medium (KeyGEN) supplemented with 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. Cells were maintained in a humidified incubator at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were collected at the end of the log phase and then counted with an automated cell counter (Bio-Rad, US) for the following experiments.

Preparation and characterization of chemically engineered cancer cells

Cells were cultured in medium containing Ac_4ManNAz (50 μM) for 72 h. After washing with DPBS, cells were incubated with DBCO-LDNA (2 μM) for 0.5 h. After washing, chemically engineered cancer cells were prepared.

To characterize if the chemically engineered cancer cells have been successfully prepared, cell imaging, zeta potential analysis and Western blotting analysis were performed, respectively. For cell imaging, DBCO-LDNA-Cy5 (2 μM) instead of DBCO-LDNA was used. The engineered A549 cells were fixed with 4% paraformaldehyde (PFA) for 10 min and imaged using a red filter. Then cell nucleuses were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 10 min and imaged using a blue filter. Fluorescent observations were performed under a LSM 710 confocal laser scanning microscope (Zeiss, Germany).

For zeta potential analysis, A549 cells treated with/without Ac_4ManNAz (50 μM) and DBCO-LDNA (2 μM) were measured using a ZETASIZER 3000HS instrument (Malvern Instruments Ltd., UK).

For Western blotting analysis, A549 cells were cultured in DMEM containing Ac_4ManNAz (50 μM) for 72 h. Cells were lysed with RIPA lysis buffer (KeyGEN, China) at 4 °C for 30 min and centrifuged at 15000 rpm for 20 min to get supernatant solution. The proteins were adjusted by bicinchoninic acid (BCA) protein assay (Sangon, China) to be 5 mg/mL. Then the azido-labeled proteins in cell lysates were biotinylated by incubation with DBCO-biotin (5 mM) in 5% bovine serum albumin (BSA) for 1 h. Then proteins were resolved by 12% SDS-PAGE gel electrophoresis and transferred to pure nitrocellulose blotting membranes (Millipore, Milford, MA, USA). After blocking with 5% BSA at room temperature for 2 h, the membranes were incubated with streptavidin-HRP at 4 °C overnight. The blotting images were obtained using a ChemiDocTM XRS Plus luminescent image analyzer (Bio-Rad, USA).

Characterization of GO presented to the cancer cells

Firstly, EDC and NHS (20 μM) were added into GO (25 μg/mL) and incubated together at 37 °C for 1 h. Then, the mixtures were further added with GFP (25 μg/mL). The products were purified using an amicon filtration device 30,000 cut-off (Millipore, USA) by centrifuge ultrafiltration. The chemically engineered cells were exposed to GFP-labeled GO at 37 °C for 1 h. After washing, cells were fixed with 4% PFA for 10 min. Then cell nucleuses were stained with DAPI, and cell membranes were stained with Dil and imaged using a red filter. Finally, the cells were analyzed using a confocal laser scanning microscope.

For atomic force microscope (AFM) characterization, LS-GO and SS-GO (25 μg/ml) were deposited on the surface of mica substrates (Yunfeng Co. Ltd., China) and dried with protection from light. AFM images were obtained by using an ex situ Agilent 5500 AFM system (Agilent, USA).

Wound healing assay

The chemically engineered cells were seeded in 6-well plates and incubated at 37 °C for 24 h. Then cells were exposed to GO (25 μg/mL) at 37 °C for 1 h. Scratches created an empty gap between the cell monolayers. After washing with DPBS, cells were cultured in serum-free medium at 37 °C for 24 h. The area of wound healing was photographed using a microscope (Nikon, Japan).

Migration assay and invasion assay

In migration assay, the chemically engineered cells were seeded in 24-well transwell chambers (Corning, China) and exposed to GO (25 μg/mL) at 37 °C for 1 h. Then cells were incubated in medium with 1% FBS at 37 °C for 24 h. Meanwhile medium containing 10% FBS was added to the lower chamber. After 24 h, the cells in the upper chamber were fixed with 4% PFA and washed with PBS. Then cells in the lower surface of the membrane were stained with 0.1% crystal violet and imaged under the microscope. For invasion assay, the chemically engineered cells were seeded in 24-well transwell chambers with matrigel (BD Biosciences), and all other steps are the same as that of migration assay.

Cytoskeletal staining

The chemically engineered cells were seeded in confocal dishes and incubated at 37 °C for 24 h. Then cells were exposed to GO (25 μg/mL) at 37 °C for 1 h. After washing with DPBS, cells were fixed with 4% PFA, treated with 1 ×phalloidin-iFluor 488 and imaged using a green filter. Then cell nucleuses were stained with DAPI. Finally, the cells were analyzed using a confocal laser scanning microscope.

Cell viability, proliferation and apoptosis

The chemically engineered cells were seeded in 96-well plates and incubated at 37 °C for 24 h. Then cells were exposed to GO (25 μg/mL) at 37 °C for 1 h. After washing, 10 μL of CCK-8 reagent was added at 37 °C for 2 h. The cell viability was assessed at 450 nm on the SpectraMax M2 Multi-Mode Microplate Reader. For cell proliferation, after treated with GO,

cells were cultured in medium containing 10% FBS. After 24, 36, 48, 60 and 72 h of incubation respectively, 10 μL of CCK-8 reagent was added to each well at 37 °C for 2 h. The cell numbers were counted on the SpectraMax M2 Multi-Mode Microplate Reader. For flow cytometric analysis of cell apoptosis, the chemically engineered cells were seeded in 6-well plates and treated with GO. Afterward, PI and Annexin V-FITC were employed for staining the cells. Finally, the cells were analyzed using CytoFLEX Flow Cytometer (Beckman Coulter, USA).

Animal experimentation

All animals received care in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals. All animal experimental procedures were performed in accordance with the ethical guidelines issued by Ethics Committee of Shanghai University (ECSHU). Female balb/c nude mice (4-5 weeks old, body weight around 20 g) were obtained from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Befor GO injection, the mice were subcutaneously inoculated with 1×10^7 A549 cells suspension (a mixture of DMEM and Matrigel in 10:1 volume ratio) under aseptic conditions. When the tumor reached about 100 mm^2, Ac_4ManNAz (40 mg/kg) was injected to mice through tail vein once daily for 3 days. Then, DBCO-LDNA (2 μM, 10 mL/kg) was injected to mice through tail vein every eight days. Afterward, GO (300 μg/ml) was implanted in the region of the subcutaneous tumor every eight days. The same amount of saline was injected to mice through tail vein as control (n = 4 per group). After 21 days, the mice were sacrificed. The organs (heart, liver, spleen, lung, and kidney) as well as tumors were collected and fixed in 4% PFA. For H&E staining, organs and tumors were embedded in paraffin blocks and then visualized using the microscope.

In vivo fluorescence imaging

After treated with saline, or Ac_4ManNAz/LDNA, or Ac_4ManNAz/LDNA/LS-GO, or Ac_4ManNAz/LDNA/SS-GO, DBCO-Cy5 (5 mg/kg) was injected to tumor bearing mice through tail vein for 1 h. The fluorescence imaging of mice, major parenchyma organs and tumor tissues were obtained and quantified with a Xenogen IVIS Lumina XRMS Series III biophotonic imaging system.

Transcriptomic sequencing and qRT-PCR analysis

Total RNA (approximately 40 μg) from unengineered A549 cells and LS-GO-treated chemically engineered A549 cells was extracted using Trizol reagent (Sangon Biotechnology, Shanghai, China). Then, the samples were sent to Shanghai Personal Biotechnology Co. Ltd. (Shanghai, China) for transcriptomic sequencing. The results were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

For qRT-PCR analysis, total RNA was isolated using the UNlQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotechnology, Shanghai, China) according to the manufacturer's instructions. The concentration and purity of total RNA was assessed by Nanodrop (Thermo Scientific, USA). Total RNA samples (1 μg per reaction) were transcribed in reverse into cDNAs using a Prime Script RT Master Mix (Takara, Japan) according to the manufacturer's instructions. qRT-PCR was then performed with the cDNA using a TB Green Premix Ex Taq (Takara, Japan) according to the manufacturer's instructions on a CFX96 Real-Time System (Bio-Rad, USA). Primer sequences were shown in Table S2.

Immunofluorescence assay of EGF, EGFR and Phosphorylated EGFR

Unengineered, chemically engineered and LS-GO-treated chemically engineered A549 cells were serum-starved overnight with 0.5% FBS, respectively. After washing with DPBS, cells were treated with serum-free DMEM containing EGF (100 ng/ml) at 37 °C for different times. After washing, cells were fixed with 4% PFA and blocked with 2% BSA. For immunofluorescence assay of EGF and phosphorylated EGFR, cells were incubated with the primary antibodies of EGF and phosphorylated EGFR (p-EGFR), respectively at room temperature for 2 h. After washing, the cells were incubated with Alexa-488 conjugated second antibody at room temperature for 1 h. The cell nucleuses were then stained with DAPI for 10 min. For visualization of EGFR, chemically engineered A549 cells using DBCO-LDNA-Cy5 instead of DBCO-LDNA were fixed with 4% PFA and blocked with 2% BSA, then incubated with the primary antibody of EGFR. Then, the cells were incubated with the Alexa-488 conjugated second antibody and stained with DAPI. Immunofluorescence assay was performed on a confocal laser scanning microscope using a green filter for Alexa-488, a red filter for Cy5 and a blue filter for DAPI.

Western blotting analysis

Cells were lysed with RIPA Lysis Buffer (KeyGEN, Nanjing, China) at 4 °C for 30 min and centrifuged at 15000 rpm for 20 min to get supernatant solution. Then the lysates (3 mg/mL) were resolved by 12% SDS-PAGE gel electrophoresis and transferred to pure nitrocellulose

blotting membranes (Millipore, Milford, MA, USA). The membranes were blocked with 5% BSA at room temperature for 2 h. Then, the membranes were incubated with primary antibodies at different dilutions at 4 °C overnight. Afterward, the membranes were incubated with secondary antibodies at room temperature for 2 h. Finally, the membranes were visualized using a ChemiDocTM XRS Plus luminescent image analyzer (Bio-Rad, United States).

Statistical Analysis

All data were expressed as the mean ± standard error. A two-tailed paired Student's *t*-test was used to compare the differences. Difference with $p < 0.05$ was considered to be statistically significant.

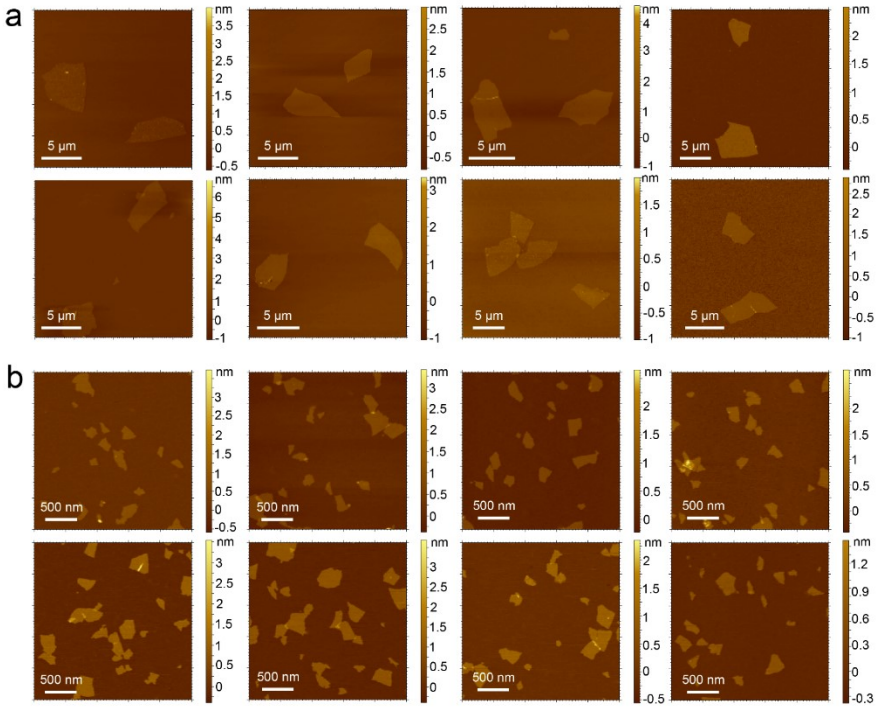


Fig. S1 Atomic force microscopic (AFM) images of (a) LS-GO and (b) SS-GO.

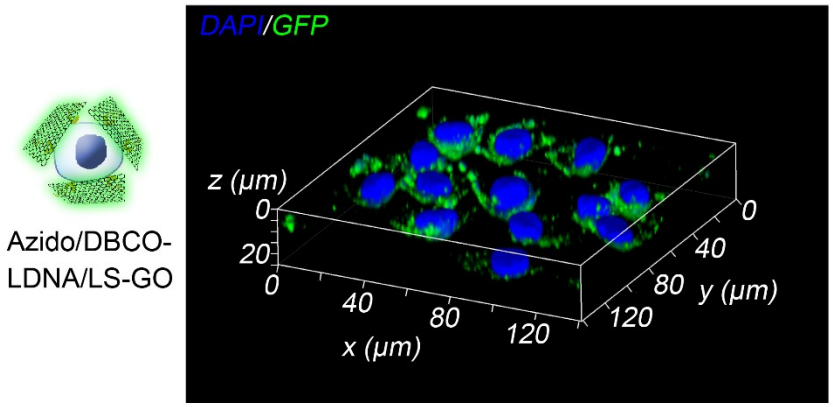


Fig. S2 The three-dimensional fluorescence image of the chemically engineered A549 cells exposed to GFP-labeled GO.

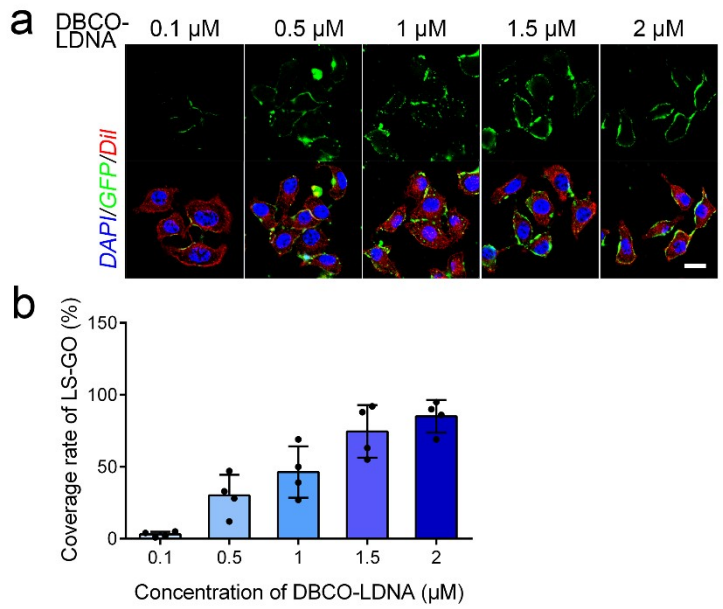


Fig. S3 (a) Fluorescence images of the A549 cells exposed to GFP-labeled LS-GO after treatment by $Ac_4ManNAz$ and different concentration of DBCO-LDNA. Scale bar = 20 μm. (b) Statistical results of the coverage rate of LS-GO on chemically engineered A549 cells obtained from (a). n = 4. Coverage rate of LS-GO = (Gray value of GFP on the cell membrane) / (Gray value of Dil on the cell membrane) × 100%.

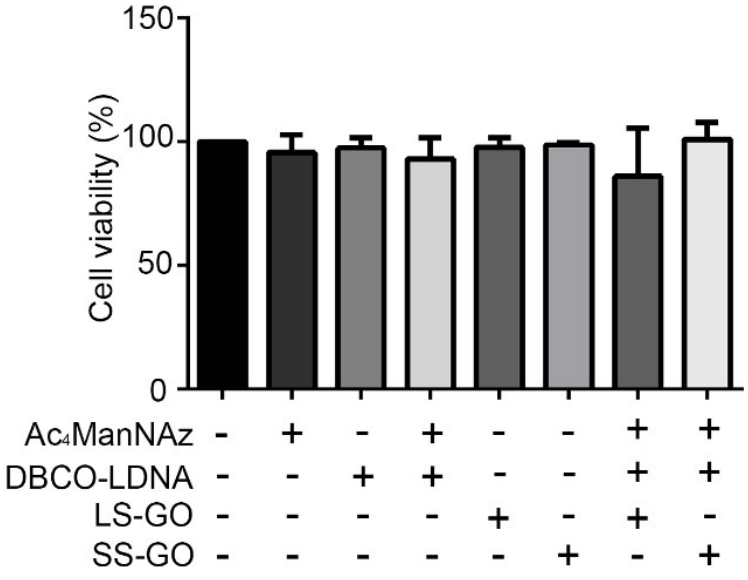


Fig. S4 The effect of the interaction between LS-GO and chemically engineered A549 cells on cell viability. n = 3.

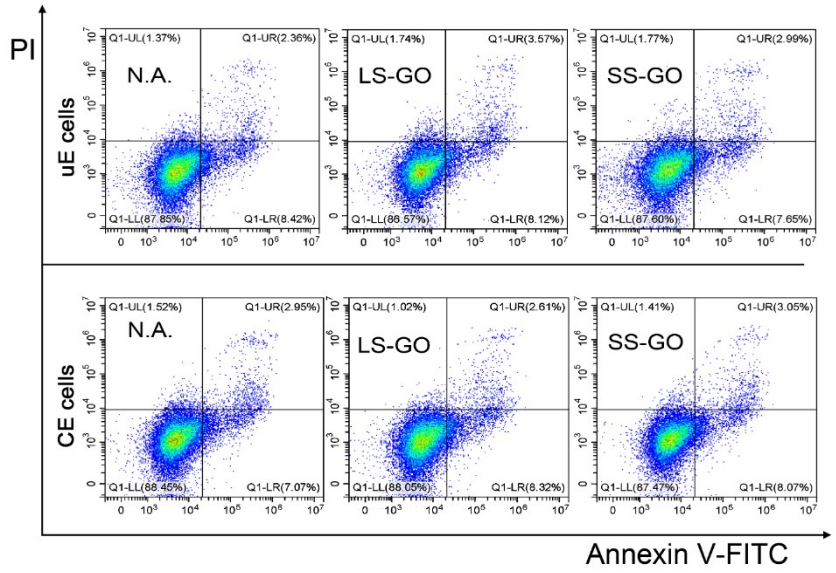


Fig. S5 The effect of the interaction between LS-GO and chemically engineered A549 cells on apoptosis. uE cells: unengineered cells. CE cells: chemically engineered cells.

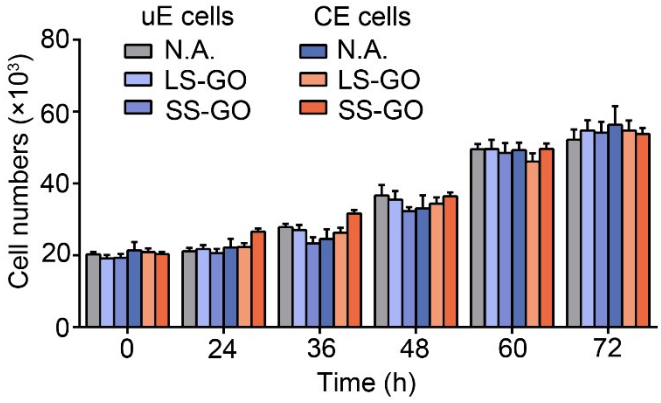


Fig. S6 The effect of the interaction between LS-GO and chemically engineered A549 cells on cell proliferation. $n = 3$. uE cells: unengineered cells. CE cells: chemically engineered cells.

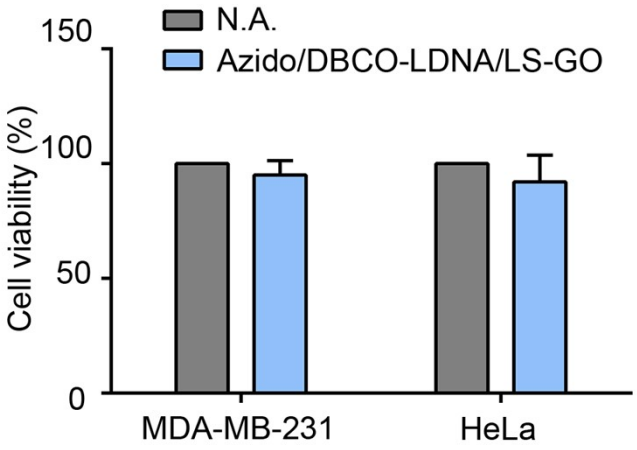


Fig. S7 The effect of the interaction between LS-GO and chemically engineered MDA-MB-231 cells / HeLa cells on cell viability. $n = 3$.

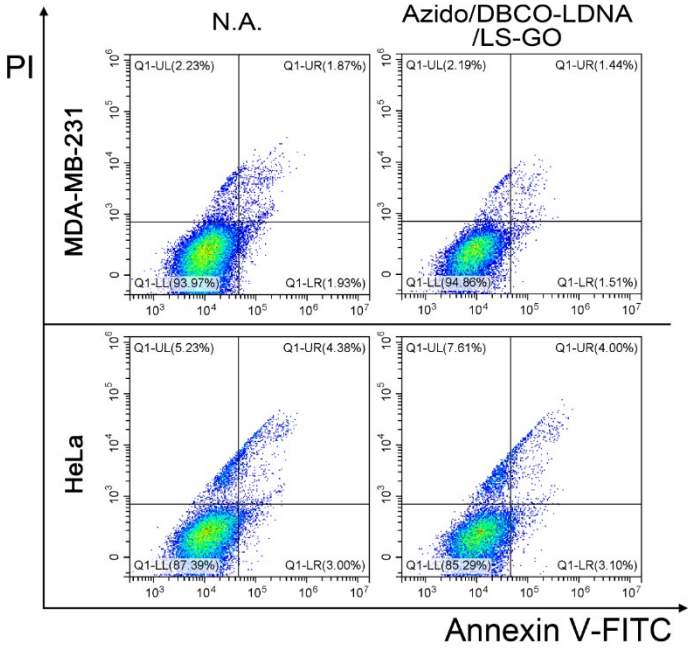


Fig. S8 The effect of the interaction between LS-GO and chemically engineered MDA-MB-231 cells / HeLa cells on apoptosis.

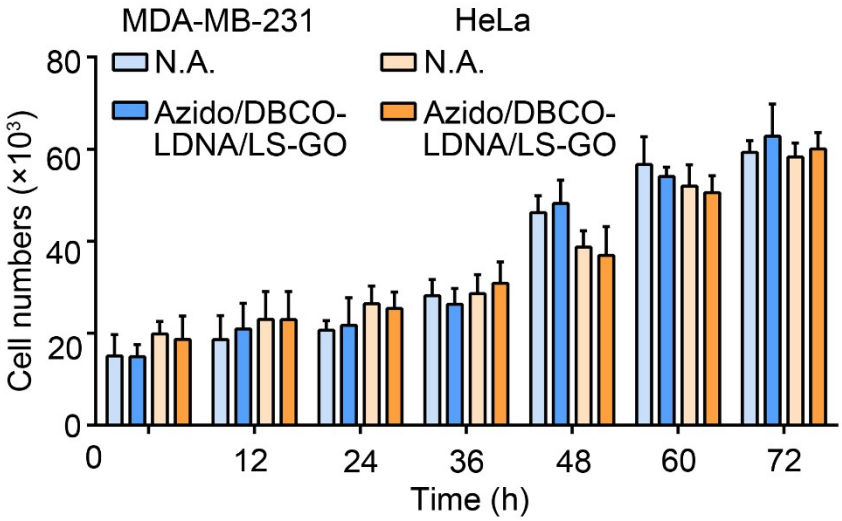


Fig. S9 The effect of the interaction between LS-GO and chemically engineered MDA-MB-231 cells / HeLa cells on cell proliferation. $n = 3$.

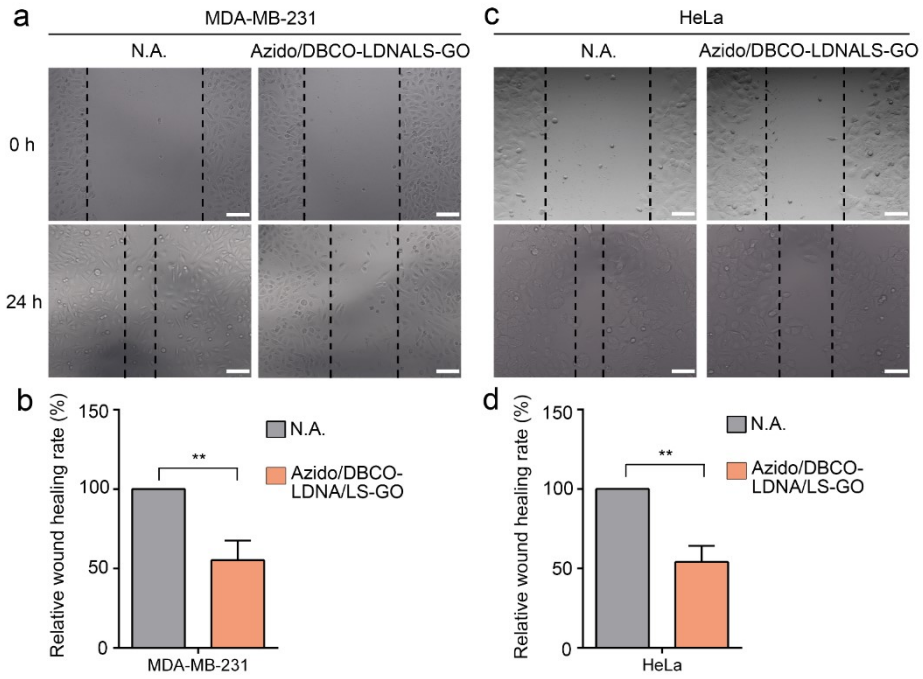


Fig. S10 Wound healing assay of the effect of LS-GO on the migration of chemically engineered MDA-MB-231 cells and HeLa cells. $n = 3$. Scale bar = 100 μm. p values were calculated by the Student's t-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

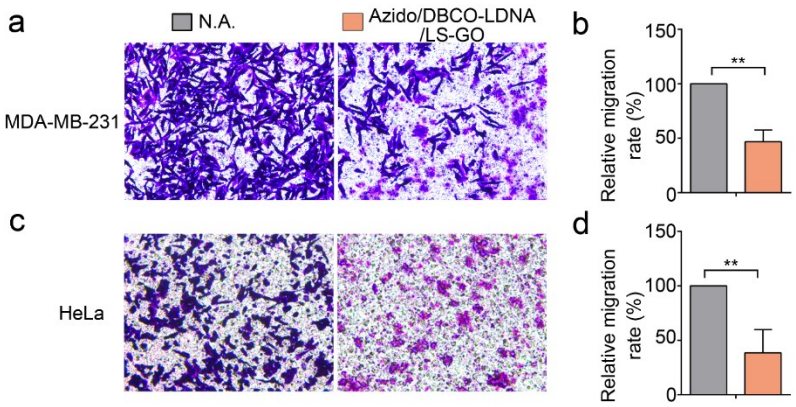


Fig. S11 Transwell migration assay and statistical results of the effect of LS-GO on the migration of chemically engineered MDA-MB-231 cells and HeLa cells. $n = 3$. p values were calculated by the Student's t-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

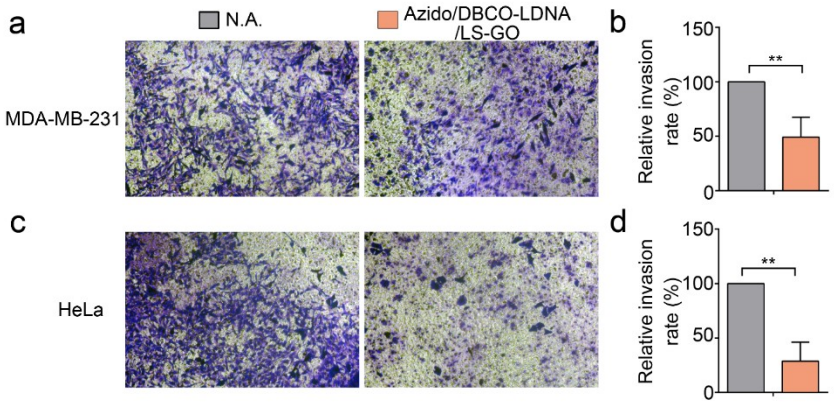


Fig. S12 Transwell matrigel invasion assay and statistical results of the effect of LS-GO on the invasion of chemically engineered MDA-MB-231 cells and HeLa cells. $n = 3$. p values were calculated by the Student's t-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

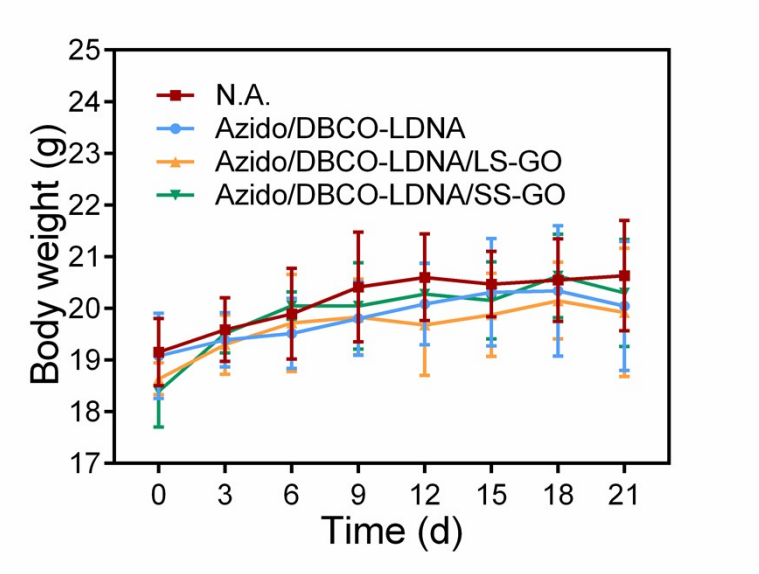


Fig. S13 Body weight changes in tumor-bearing mice during different treatments. $n = 4$.

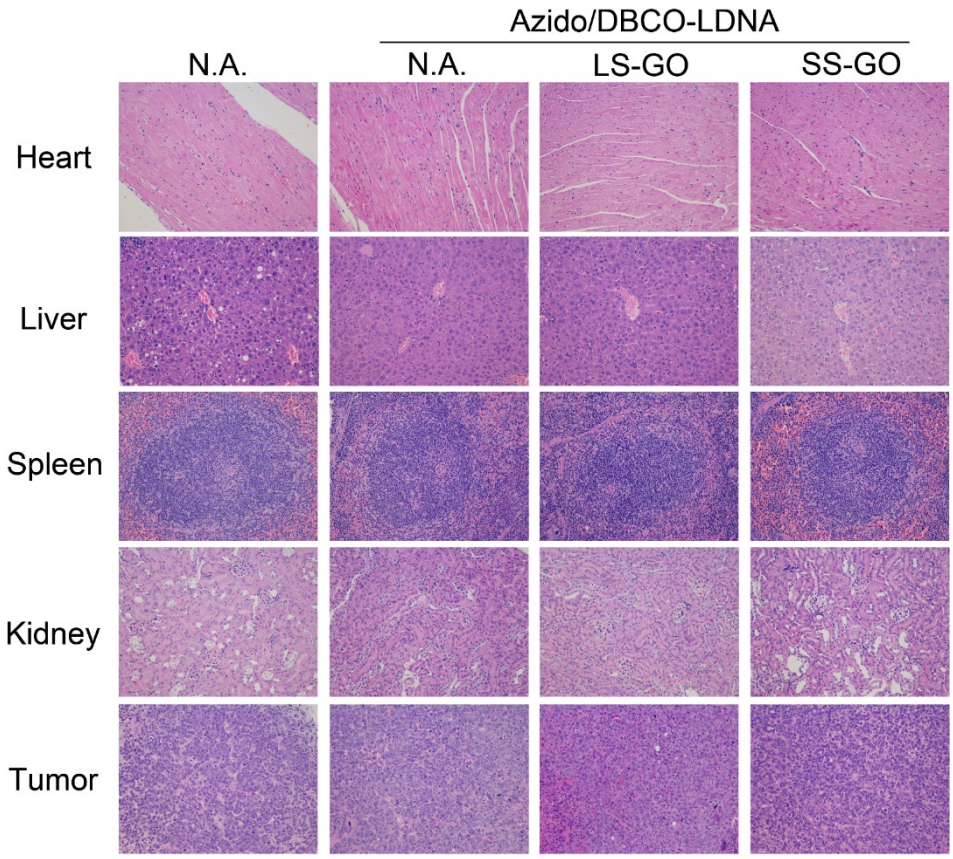


Fig. S14 H&E staining images of some organs and the subcutaneous tumors collected from the mice with different treatments. $n = 4$. Original magnification: 20 ×.

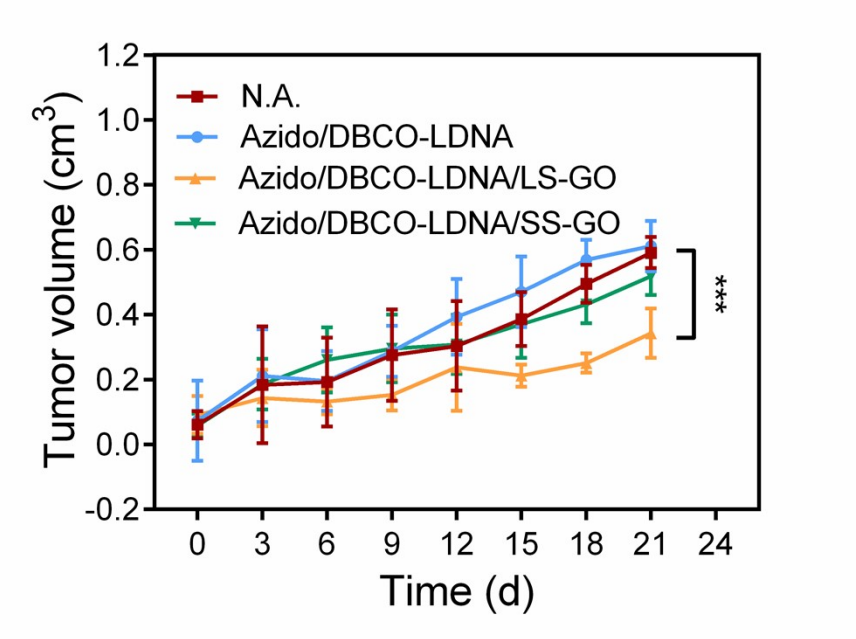


Fig. S15 Tumor growth curves (tumor mass volume) of the subcutaneous tumors after different treatments. $n = 4$. p values were calculated by the Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table S1 Sequences of oligonucleotides.

Oligonucleotides	Sequence (5'-3')
DBCO-LDNA	DBCO-CAGAGTCAAGAGGAGTGAGTCAGAGTCAAGAGGAGTGAGT
DBCO-LDNA-Cy5	DBCO-CAGAGTCAAGAGGAGTGAGTCAGAGTCAAGAGGAGTGAGT-Cy5
Complementary LDNA	ACTCACTCCTCTTGACTCTGACTCACTCCTCTTGACTCTG

Table S2 Primer sequences used for qRT-PCR.

Gene	Sequence (5'-3')
EGR1	CCCCGCCCCCGCGATGGAGAAGAA
	CCCCGCCCCAGATATCGAGAAGAA
DUSP6	CAGTGGTGCTCTACGACGAG
	GCAATGCAGGGAGAACTCGGC
FOS	CCAGTCAAGAGCATCAGCAA
	AAGTAGTGCAGCCCGGAGTA
JUN	ACTCGGACCTTCTCACGTCG
	TAGACCGGAGGCTCACTGTG
β-actin	AGCCATGTACGTAGCCATCC
	CTCTCAGCAGTGGTGGTGAA

Figure 5e

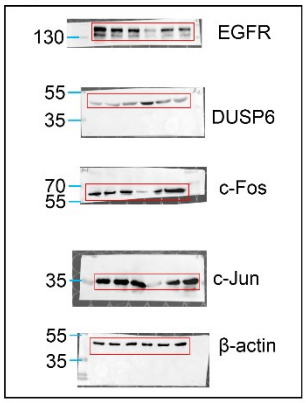
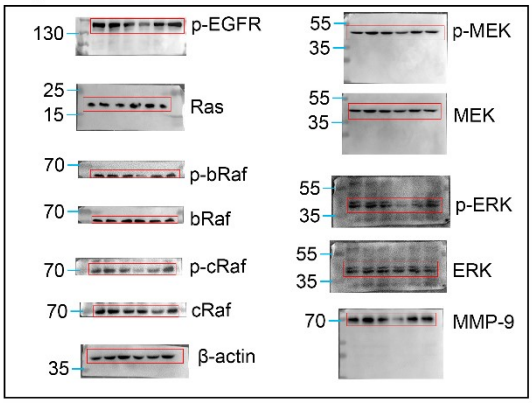


Figure 6a



Raw data