SiRNA Delivery with PEGylated Graphene Oxide Nano-sheets for Combined Photothermal and Genetherapy for Pancreatic Cancer

Feng Yin[a#], Kuan Hu[a#], Yangzi Chen[a], Mengying Yu[a], Dongyuan Wang, Qianqian Wang[a], Ken-Tye Yong[b], Fei Lu[a]*， Yongye Liang[c]* and Zigang Li[a]*

[a]School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, 518055, China

[b]School of Electrical and Electronic Engineering, Nanyang Technological University, Singapore 639798, Singapore

[c] Department of Materials Science and Engineering, South University of Science and Technology of China, Shenzhen, 518055, China.

# These authors contributed equally to this work

*Corresponding authors:

Emails: Zigang Li, email: lizg@pkusz.edu.cn

Fei Lu, email: lufei@pkusz.edu.cn,

Yongye Liang, email:liangyy@sustc.edu.cn

Supplementary Information

Supplementary Information

Materials and Methods

Chemicals and reagents

NH_2-mPEG-NHBoc (5K), dichloromethane (DCM), N,N'-dicyclohexylcarbodiimide (DCC), triethylamine (TEA), sodium bicarbonate, trifluoroacetic acid (TFA), Folic acid (FA), N-Hydroxysuccinimide (NHS), dimethyl sulfoxide (DMSO), diethyl ether, Poly(allylamine hydrochloride) (PAH, molecular weight 15,000) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromides (MTT) were purchased from Sigma Aldrich. Ultrapure water produced with a Milli-Q Integral 5 system was used in all experiments. Antibodies against HDAC1 (ab53091), K-Ras (ab55391), H3K4me2 (ab32356), H4K12ac (ab1238) and Actin (ab8227) were obtained from Abcam; antibody against Histone H4K16ac (07-329) was obtained from Millipore.

Synthesis of graphene oxide

GO was prepared by a modified Hummers method [1]. Graphite flakes (1 g) were added into a 500 ml round bottom flask, followed by the addition of 23 ml of concentrated sulfuric acid. The mixture stirred at room temperature for one day. Next, the reaction mixture was heated in an oil bath at 40°C. $NaNO_3$ (600 mg) was added to the suspension and allowed to dissolve for several minutes. The $KMnO_4$ (3 g) was added slowly in portion, keeping the reaction temperature below 45°C. The solution was then stirred for 30 minutes, followed by the addition3 ml of ice water for two times. After 5 minutes, 40 ml of ice water was slowly added. By further stirring for another 16 minutes, the flask was removed from the oil bath and 140 ml of ice water was added, followed by 10 ml of 30% H_2O_2. The reaction mixture was centrifuged to collect the precipitate and washed with 5% HCl solution and copious amounts of water. The final precipitate was dispersed in 100 ml of water and bath sonicated for 40 min. A brown supernatant was collected by a centrifugation at 5000 rpm for 5 minutes. GO solid was obtained by lyophilizing the solution.

NMR spectroscopy

NMR data were recorded on a Bruker AVANCE III 500 MHz spectrometer. Acetonitrile-d was used for ^{1}H NMR to characterize the FA-conjugated PEG. NMR data were processed using Topspin 3.0.

The molecular weight of FA-conjugated PEG was tested using the integrated product peak areas in MALDI-TOF (matrix-assisted laser desorption/ionization–time-of-flight) mass spectrometry (Applied Biosystems 4800 Plus).

FTIR spectroscopy

The biocompatible GO/FA/PEG nanocarrier was characterized with a Bruker IFS 66 v/s infrared spectrometer (Bruker, Karlsruhe, Germany) by Fourier transform infrared (FT-IR) spectroscopy in the range 400-4000 cm^{-1} with a resolution of 4 cm^{-1}. The KBr pellet technique was adopted to prepare the samples for recording the IR spectra

TGA analysis

Thermogravimetric analysis (TGA) results were obtained with a TA Instrument 2050 thermogravimetric analyzer at a heating rate of 10℃ min-1 from 25 to 800℃ under a nitrogen atmosphere. The TGA curves of the functional graphene nanosheets were shown in Figure S2D. Thermogravimetric analysis (TGA) were used to characterize the thermal properties of GO-PEG-FA. For GO, a weight loss of 22 wt% at about 80℃ and 25 wt% at about 220℃ can be observed, which can be assigned to the loss of adsorbed water on GO and groups on GO such as hydroxyl and carboxylic groups, respectively. In the case of GO-PEG-FA, the initial degradation of 18% mass loss at about 180℃ may be due to the residual oxygen functional groups on GO, the formed amide groups between GO and PEG-FA and the decomposition of the FA. The weight loss of 23% GO-PEG-FA at around 400℃ is presumably assigned to the decomposition of the PEG in GO-PEG-FA. This also indicated that the PEG-FA was bound onto GO.

Gel retardation assay to access the quantity of siRNA delivered by PEGylated GO-based nanocarriers

The siRNA binding ability of the PEGylated GO-based nanocarriers was studied by

agarose gel electrophoresis [2]. GO/PEG/PAH (1 mg/mL) or GO/FA/PEG/PAH was mixed with siRNA (130 μg/mL) at five different mass ratios (GO:siRNA= 0.5 μg: 5 μg, 1 μg: 5 μg, 2 μg: 5 μg, 4 μg: 5 μg, 8 μg: 5 μg and 10 μg: 5 μg) for 30 min at room temperature. Electrophoresis was carried out on an 1% agarose gel with a current of 100 V for 15 min in a nucleic acid electrophoresis buffer (TAE) buffer solution (40 mM Tris-HCl, 1 % acetic acidv/v, and 1 mM ethylene diamine tetraacetic acid (EDTA). The retardation of the complexes was visualized by staining with Golden View and then analyzed on a UV illuminator to show the position of the complex siRNA band relative to that of naked siRNA. The final mass ratio between GO complex and siRNA is 1μg:1μg and the final concentration of siRNA is 50 nM [3] for all experiments.

Tumor cell line and culture

Human pancreatic cancer cell line, Miapaca-2 (CRL-1420, American Type Culture Collection), were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 2.5% (v/v)horse serum (Gibco) and penicillin/streptomycin (100 μg/mL, Gibco). Liver-derived cell lines QSG-7701 (Institute of Cytology, Chinese Academy of Sciences, Shanghai, China) were maintained in DMEM medium (Gibco) supplemented with 10% (v/v) FBS (Gibco). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

siRNA transfection

The day before transfection, cells were seeded onto 6-well plates in DMEM medium with 10% FBS to give 30% − 50% confluence at the time of transfection. GO/PEG/PAH or GO/PEG/FA/PAH nanosheets dispersion (1 mg/mL, 6 μL) was mixed with a G12C mutant K-Ras $siRNA^{Cy3}$(130 μg/mL, 10 μL) or HDAC1 $siRNA^{FAM}$ with gentle vortex and left undisturbed for 30 min（Table S1）. Before transfection, the culture medium was replaced with OPTI-MEM (950 μL, Invitrogen), the above mentioned PEGylated GO/siRNA mixture was then added to the medium and the cells were continuously cultured. Four hours later, DMEM medium (500 μL)

with 30% FBS was added to the medium. Free siRNA^{FAM} or siRNA^{Cy3} was also used in another parallel experiment at the same dosage level. The gene expression was monitored at 48 hours post-transfection. For transfection efficiency examination, fluorescent imaging and flow cytometry assays were performed at 4 hours post-treatment.

Flow Cytometry

For the flow cytometry of transfection efficiency experiments, Miapaca-2 cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The FAM served as the luminescent marker (filter set for FITC was applied) and Cyc3 served as the luminescent marker (filter set for PE was applied) to determine the transfection efficiency quantitatively. The samples were analyzed using a FACS caliber flow cytometer (Becton Dickinson, Mississauga, CA). The transfection efficiency was calculated based on the reported methods (ref [2, 4]) by calculating the number of cells in different gates.

Apoptosis assay

The apoptosis assay was conducted using an Annexin V: FITC Apoptosis Detection Kit I (BD Pharmingen™) according to the manufacturer's instructions. Briefly, approximately 1×10^6 MiaPaCa-2 cells were seeded in a six-well plate. Then, the cells were treated with the different GO-based nanoformulations, and incubated for 48 hours. The cells were then collected and washed twice with PBS and suspended in binding buffer. The induction of the apoptosis process enables the FITC-labeled Annexin V to bind with phosphatidylserine (PS) as it appears on the outer surface of the cell membrane due to the onset of apoptosis, and is otherwise confined to the inner boundary of the cell membrane in healthy cells. The cell nuclei were stained with propidium iodide (PI). The stained cells were analyzed by flow cytometry to determine the apoptotic cells. The cells with positive fluorescence intensity signals for both FITC and PI were used for the apoptotic cell count.

Cell cycle analysis

For the flow cytometry of cell cycle arrest experiments, Miapaca-2 cells were washed twice with PBS and harvested by trypsinization. The cells were fixed with cold 70%ethanol for 4 hours and followed by centrifugation at2000 rpm for 5 min to remove the ethanol. Then the fixed cells were dispersed in PBS with 1% Triton-100,1 mg/mL RNase and 5 mg/mL PI, stained at 37ºC for30 min. The samples were analyzed by flow cytometer and the percentages of cells in G0/G1, S, and G2/M phases were determined by FlowJo software.

RNA isolation and quantitative RT-PCR

48 hours after transfection, total RNA was extracted from cells using TRIzol reagent (Invitrogen) and the amount of RNA was quantified by a spectrophotometer (Nano-Drop ND-2000). Total RNA (2 μg) was reverse transcribed to cDNA using the reverse transcriptase kit from Promega according to the manufacturer's instructions. The mRNA levels of the target genes were quantified by real time PCR using SYBR green (Promega) in an ABI Prism 7500 real-time PCR system (Applied Biosystems). Primers were shown in Table S2.

Western Blotting

For western blot analysis, cells were seeded in 6-well plates and treated for 24 hours with GO-based nanocarriers as described for the RT-PCR assay. To isolate protein, cells were washed with PBS and harvested using the lysis buffer (50 mM Tris·Cl PH=6.8, 2% SDS, 6% Glycerol, 1% β-mercapitalethanol, 0.004% bromophenol blue). Total cellular protein concentrations were determined by a spectrophotometer (Nano-Drop ND-2000). 20 μg of denatured cellular extracts were resolved by 10% SDS-PAGE gels. Protein bands in the gel were then transferred to Nitrocellulose Blotting membranes and incubated with the appropriate primary antibody. The antibody dilutions were as follows: 1:1000 for K-Ras, 1:1000 for HDAC1, 1:1000 for H4K16ac, 1:1000 for H3K4me2, 1:1000 for H4K12ac and 1:1000 for actin. Membranes were incubated overnight at 4 °C and washed the next day with buffer (1xPBS, 0.05% Tween 20). Goat anti-rabbit or anti-mouse secondary antibodies were used for secondary incubation for 1 hour at room temperature. Proteins were then

visualized with chemilluminescent substrates.

Cell viability test

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylt -etrazolium bromide, Sigma) assay. Cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and incubated with different PEGylated GO nanoformulations for indicated time points. For combination with NIR light, Miapaca-2 cells were incubated with FA/GO nanosheets or FA/GO/si(HDAC1+Kras) RNA nanoformulations for 4 hours, washed with PBS twice, and then irradiated by a 808 nm NIR light at power densities of 0.5, 1 or 2 W/cm^2 for 2 minutes. MTT (5 mg/mL, 20 μL) in PBS was added and the cells were incubated for 4 hours at 37 °C with 5% CO_2. DMSO (Dimethylsulfoxide, 150 μL, Sigma) was then added to solubilize the precipitate with 5 min gentle shaking. Absorbance was measured with a microplate reader (Bio-Rad) at a wavelength of 490 nm. The cell viability was obtained by normalizing the absorbance of the sample wells against that from the control wells and expressed as a percentage, assigning the viability of non-treated cells as 100%.

In vivo hemocompatibility

The protocol for in vivo hemocompatibility was based on previous literatures [5]. The general procedure was showed here: Fresh ethylenediamine tetraacetic acid (EDTA)-stabilized human whole blood samples were taken from animal center. Typically, 5 mL of whole blood was added to 10 mL of calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS) and centrifuged at 500 g for 10 min to isolate RBCs from serum. This purification step was repeated three times, and then the washed RBCs were diluted to 50 mL in PBS. To test the hemolytic activity of GO/FA/SiRNA and GO/PEG/SiRNA samples, 0.1 mL of diluted RBC suspension (around 1 x 10^8 cells mL^{-1}) was added to 0.2 mL of GO/FA/SiRNA and GO/PEG/SiRNA suspension solutions in PBS at different concentrations. The final concentration of GO/FA/SiRNA and GO/PEG/SiRNA ranges from 12.5 to 3200 μg mL^{-1}. D.I. water (+RBCs) and PBS (+RBCs) were used as the positive control and negative control, respectively. All the samples were placed on a rocking shaker in an incubator at 37° C for 3 h. After incubation, the samples were centrifuged at 12000 rpm for 5 min. The hemoglobin absorbance in the supernatant was measured at 540 nm, with 655 nm as a reference, using an iMark microplate reader (BioRad, Hercules, CA).

Mice voluntary cage-wheel exercise

BALB/c mice (female; 6 weeks old) were obtained from Vital River Laboratory Animal Technology Co. Ltd. of Beijing, People's Republic of China and allowed an acclimation period of 1 week at 22 ± 2 °C with a 12-hour light: dark cycle (lights on 8am, lights off 8pm). Subsequently, BALB/c mice were randomly divided into 2 groups (3-4 mice per group) and were subcutaneously injected with PBS or FA/GO (50 mg/kg). Voluntary running was performed by these two groups at the start of exercise followed with the reported protocol [6]. The voluntary running system consisted of six separated chambers (Chengdu TME Technology Co., Ltd, China) was used in the animal performance study. During the training session, mice were placed on the motorized rod (30 mm in diameter) in the chamber. The rotation speed gradually increased from 0 to 100 rpm over the course of 100s. The rotation speed was recorded when the animal fell off from the rod. Each rotarod training session consisted of 7 trials and lasted around 7 minutes. Performance was measured as the average rotation speed animals achieved during the training session. The two different groups were all trained in five same continuous time point (day 0, day 2, day 4, day 8, day 12, day16 and day 20). No significant differences were found between the two groups.

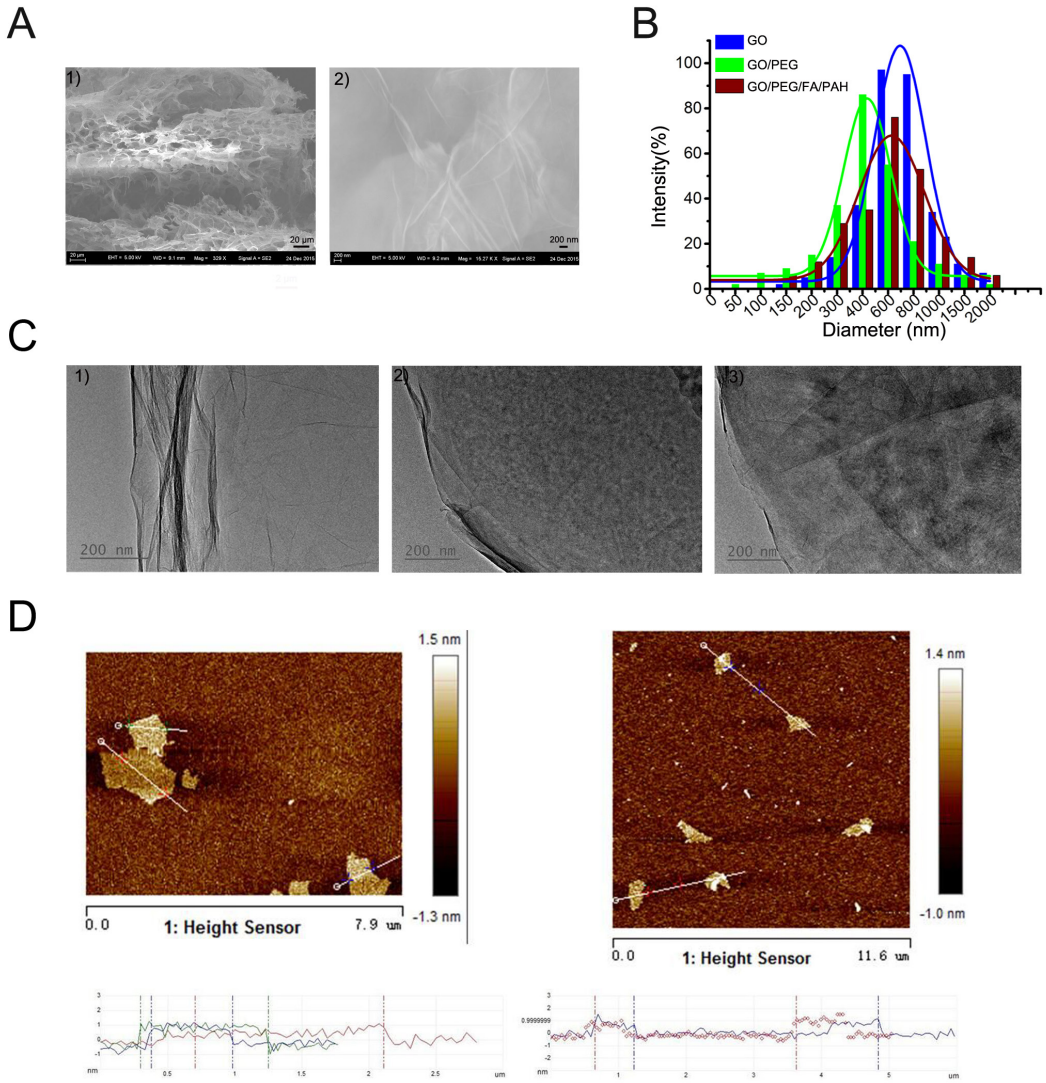
In vivo imaging

When the tumor reached an appropriate volume of 200–300 mm^3, the mice were injected with 200 μL of FA/GO/siRNA^{Cy3} (contained both K-Ras siRNA^{Cy3} and HDAC1 siRNA^{Cy3}, FA/GO 4mg/kg, siRNA 40μg=2.5 nmol, injection) by tail vein injection. After injection, mice were anesthetized with isoflurane. The induction concentration was 5% isoflurane/1L O_2, and the maintenance concentration was 2–3% isoflurane/1L air. Once the mice were properly anesthetized, they were imaged at indicated time points to monitor the accumulation of FA/GO/siRNA^{Cy3} nanoparticles in tumors using the IVIS LuminaII small animal in vivo optical imaging system (Caliper). In this study, the scanning wavelength range between 500 and 950 nm was used for in vivo imaging.

Preparation of paraffin section histological analysis (IHC)

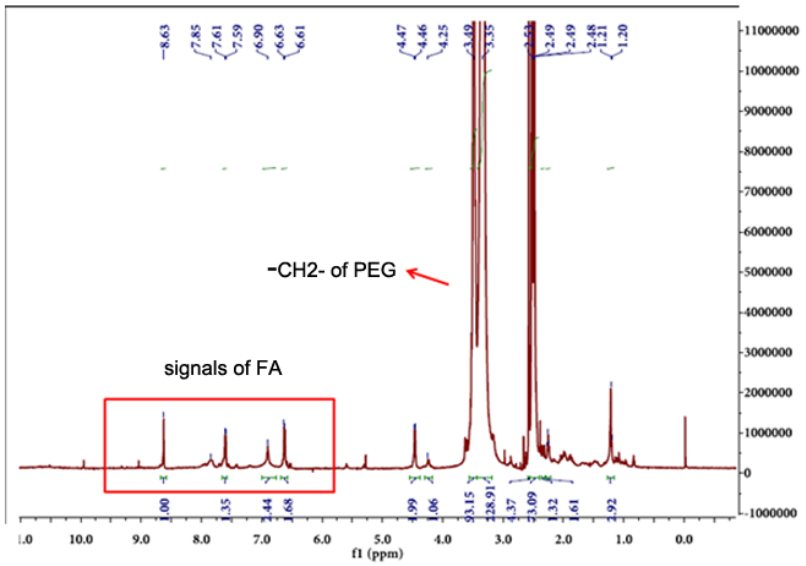
For histological experiments, organ tissues were collected on the final day and fixed in 4% buffered formalin-saline at room temperature for 24 hours. Following this, tissues were embedded in paraffin blocks and paraffin sections of 4 mm thickness were mounted on a glass slide for hematoxylin and eosin (H&E) staining. The H&E staining slices were examined under a light microscopy (Olympus BX51).

For immunohistochemistry assay, pancreatic carcinoma tissue microarray slides were immersed in 3% H_2O_2 for 5 minutes to inactivate the endogenous peroxidase. Nonspecific binding sites were blocked using 5% BSA for 15 minutes. Antibodies against HDAC1, K-Ras, P53, Caspase-3 or RhoB were diluted as the primary antibodies and were incubated with slides at 4 ^{o}C overnight, washed and then incubated with Rabbit-Probe or Mouse-Probe $MACH_3$ HRP-polymer detection system according to the supplier's instructions. Slides were developed with 3, 3'-diaminobenzidine substrate using the ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories) for 1-5 minutes, counterstained with hematoxylin. (All reagents were obtained from Biocare Medical.)

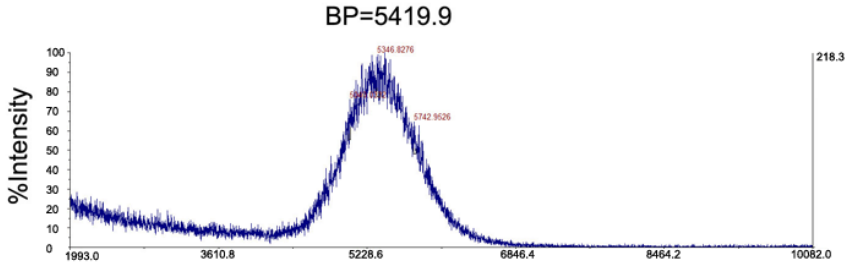


Supplementary Figure S1. (A) Scaning electron microscope of the monolayer 2D GO nanosheets. (B) Statistics of sheet size of GO nanosheets before and after PEGylation based on dynamic light scattering (DLS) measurements. The decrease of PEGylated GO nanosheet sizes was also verified. (C) Transmission Electron Microscope of the monolayer 2D GO nanosheets: 1) bared GO; 2) PEG/GO; 3) FA/PEG/GO. (D) AFM data was further analyzed to show the height information of particles of bared GO and PEGylated GO nanosheet.

A


-CH2- of PEG
signals of FA
f1 (ppm)

B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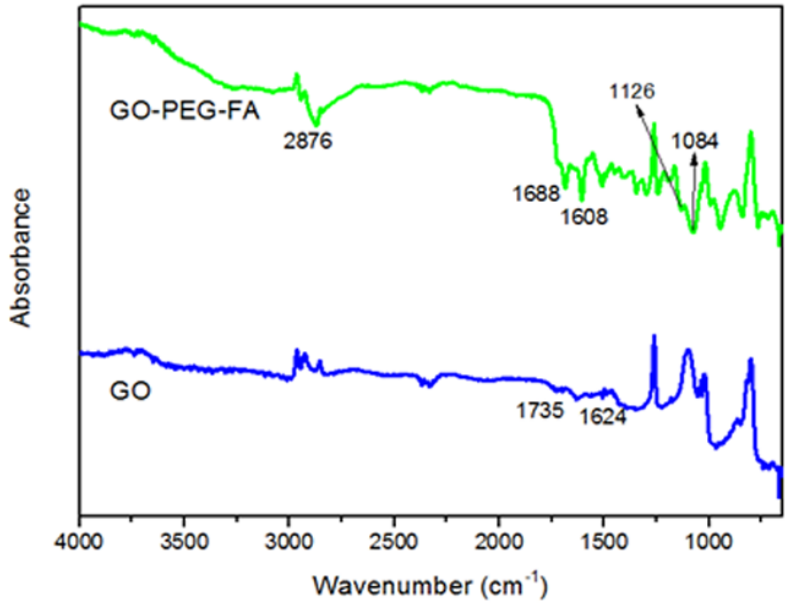
10082.0

C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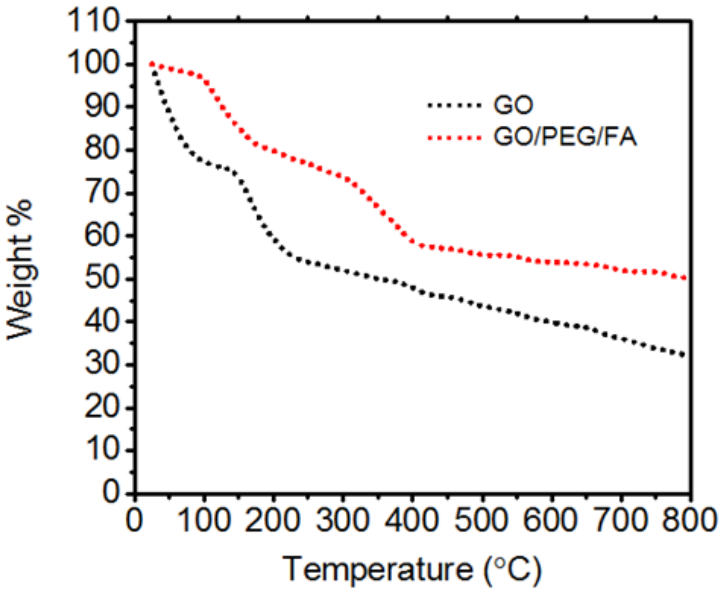
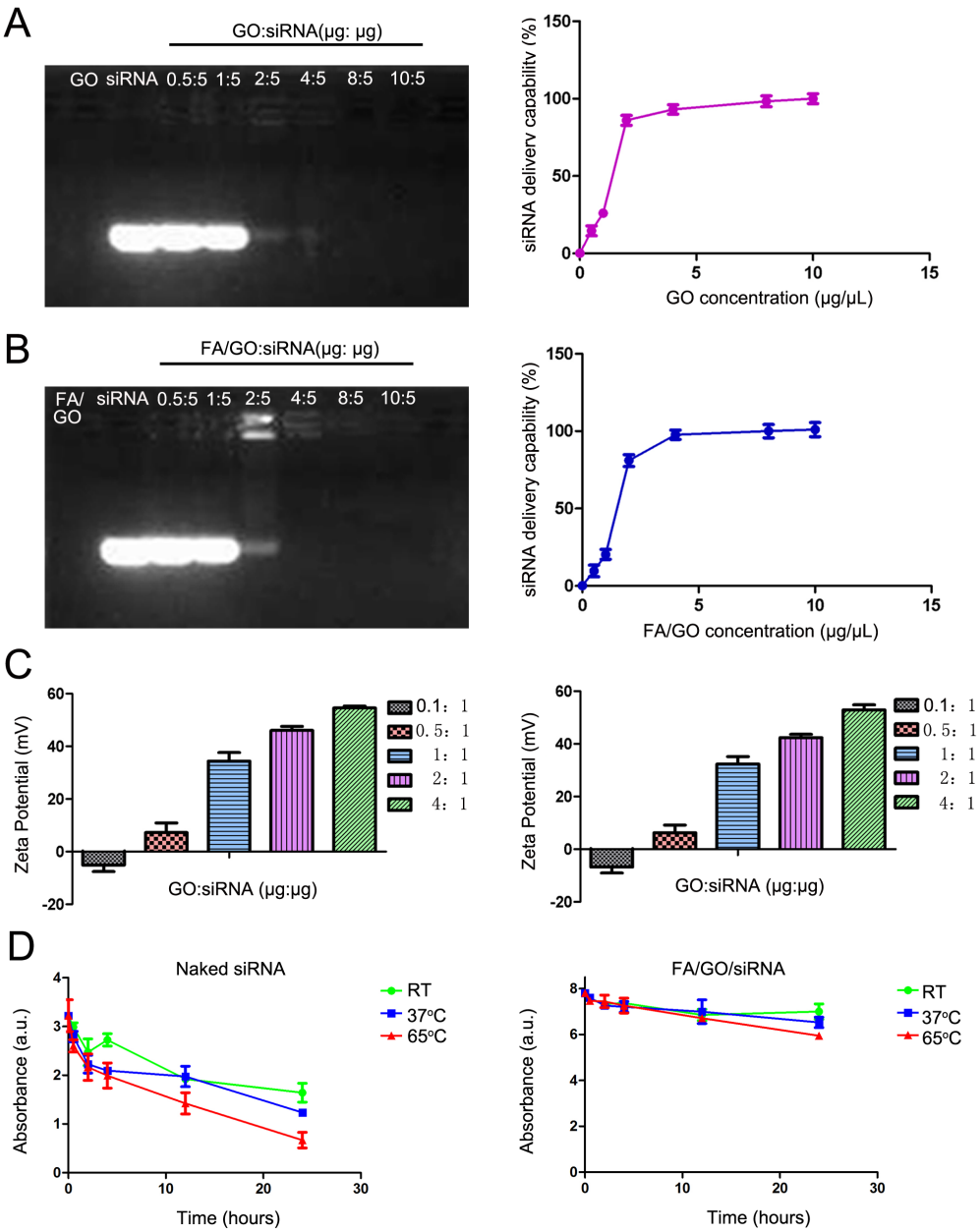
1126
1084
1688
1608
GO
1735
1624
Absorbance
Wavenumber (cm-1)

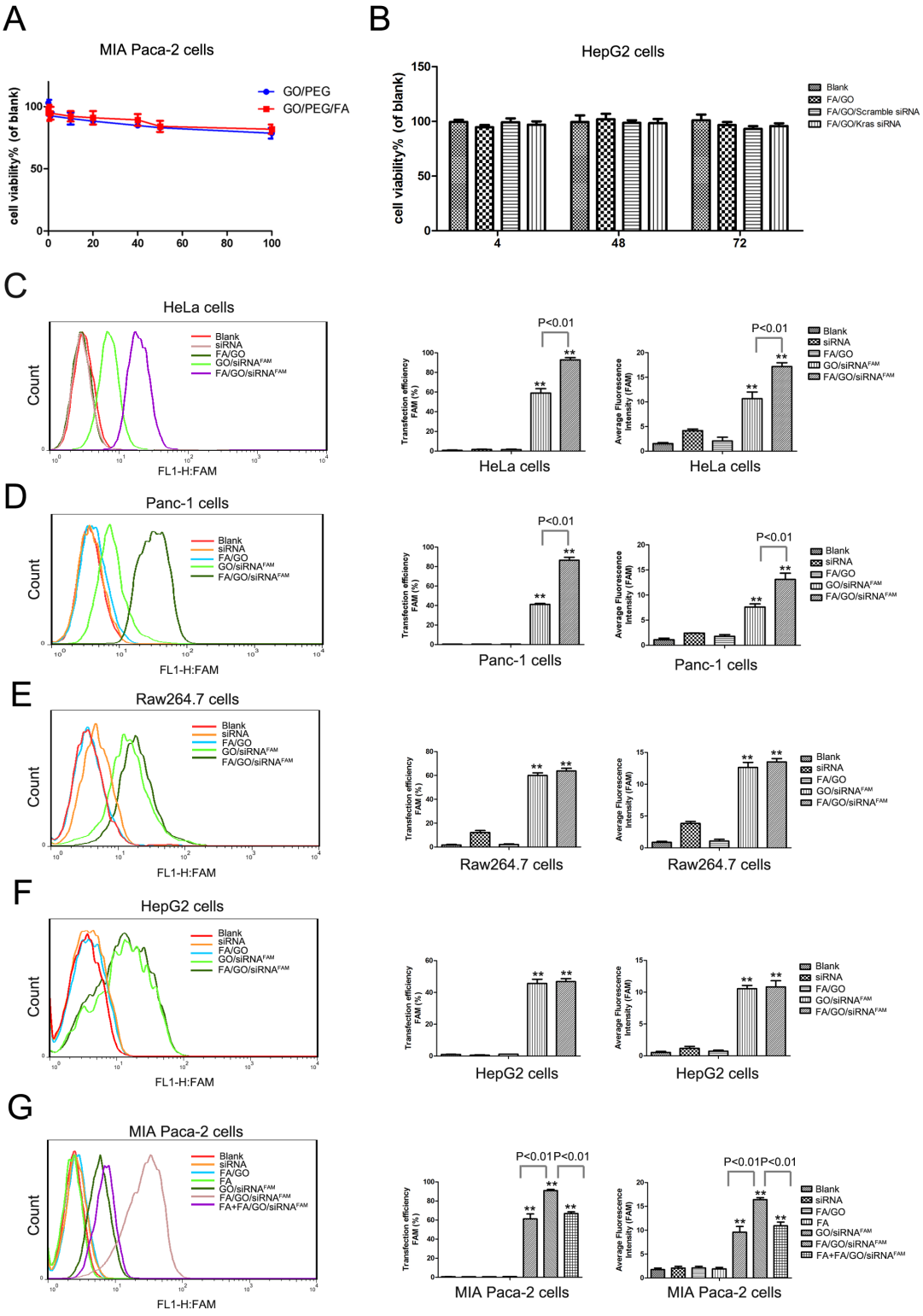
D



Supplementary Figure S2. (A) ^{1}H NMR of folic acid conjugated to PEG5000 (in deuterated acetonitrile). The δ=3.51ppm was marked as the -CH2- of PEG, the peaks at δ=6.0–6.5 ppm attributed to FA. (B) The MADLI-TOF MS results of FA-PEG5000-NH2. (C) FT-IR spectra of the GO and GO/PEG/FA nanosheets measured in KBr pellets. (D) TGA curves of the GO and GO–PEG–FA nanosheets under a N2 atmosphere.

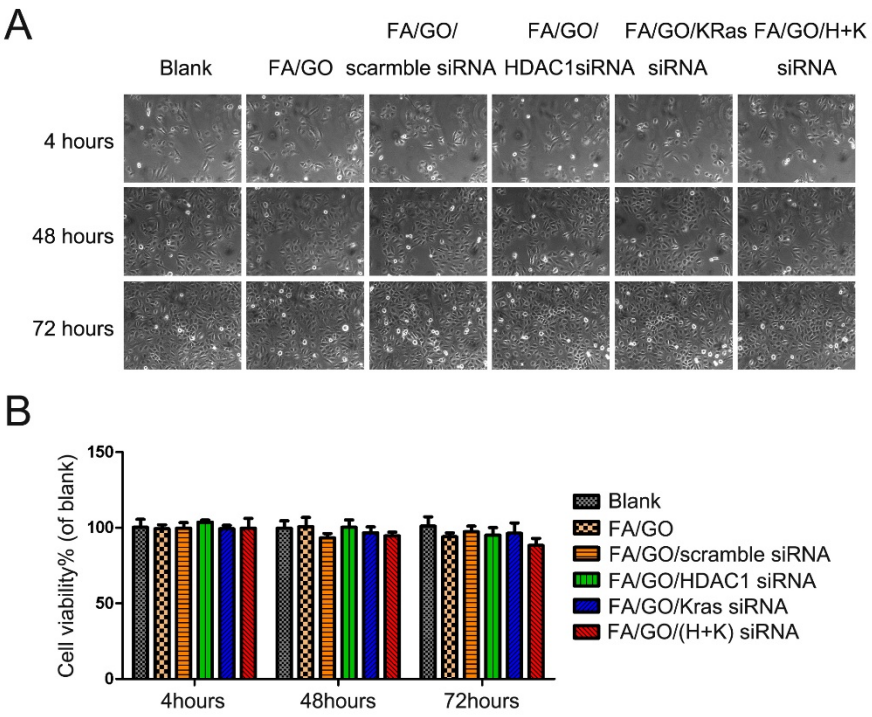


Supplementary Figure S3. Optimization of GO/siRNA ratio and zeta potential measurement. (A, B) Gel retardation to optimize the mass ratio between FA/GO and siRNA. (C) Zeta potential detection at different incubation ratio between GO/PEG/PAH (GO) or GO/FA/PEG/PAH (FA/GO) and siRNA. With the GO: siRNA ratio enlarge, the zeta potential of the nanocomplex increases.(D) siRNA stability test in the GO formulation. Naked siRNA or FA/GO/siRNA nanocomplex were incubated in DMEM medium containing 10% FBS at room temperature (RT, 25°C), 37 °C or 65 °C for designated time interval (0, 0.5h, 1h, 4h, 12h and 24h). Absorbance data of naked siRNA or FA/GO/siRNA nanocomplex at OD260 were detected by Nanodrop 2000.

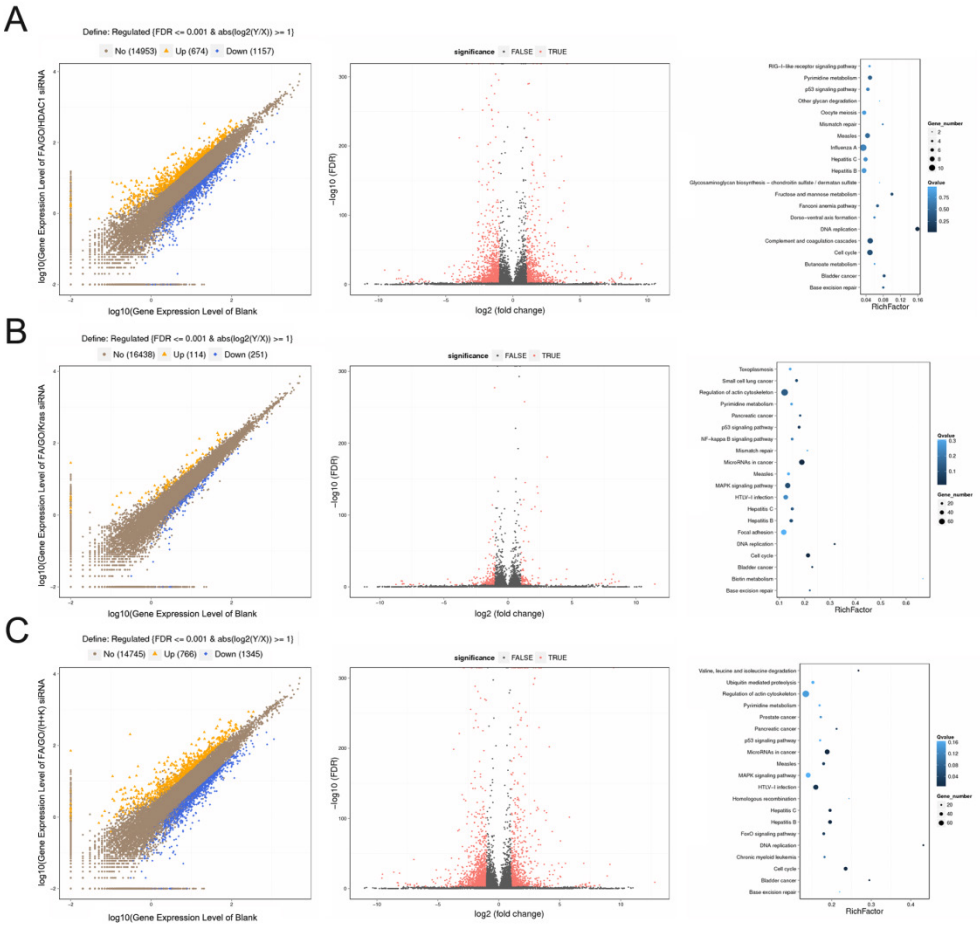


Supplementary Figure S4. (A) Viability of cells after incubation with GO/PEG or GO/PEG/FA at variation concentrations up to 100uM for 48hours. Both the GO/PEG and GO/PEG/FA complex showed negligible cell growth inhibition effect. (B) Relative cell viabilities of MIA PaCa-2 cells treated with PBS, FA/GO,

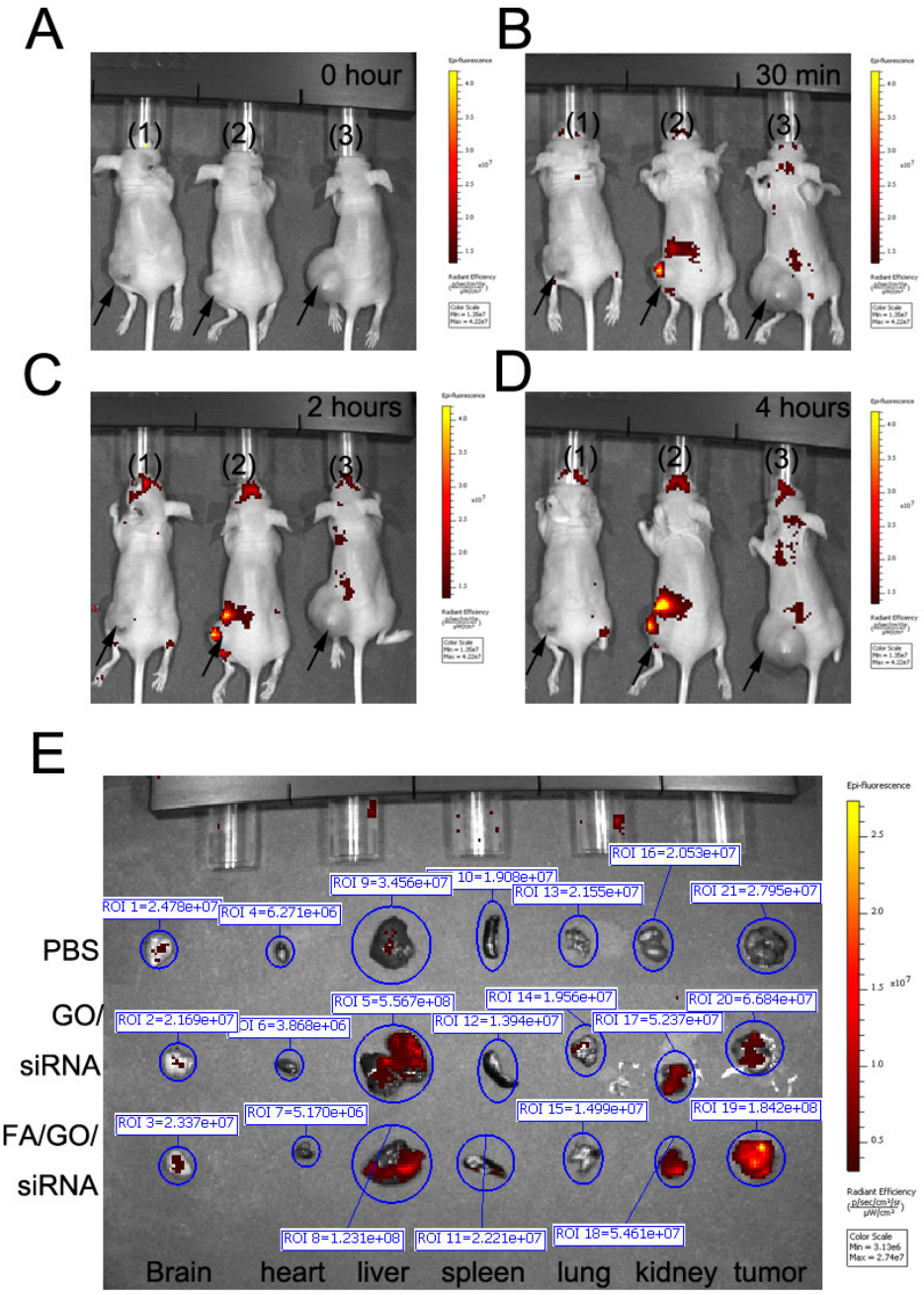
FA/GO/scramble siRNA and FA/GO/K-Ras siRNA for 4 hours, then washed with PBS and re-incubated in fresh cell medium for the designated period of time. (C-G) Flow cytometry evaluations on the transfection efficiencies. (G) MIA Paca-2 were pre-treated with 30 mg/mL FA for 30 minutes to block the FR on the cellular surface [4] and then the samples were incubated with FA/GO/siRNA^{FAM} nanocomplex for 4 hours. Data are presented as the mean±SEM of triplicate experiments.*, $P < 0.05$, **, $P < 0.01$ vs PBS (as blank), siRNA^{FAM} and FA/GO.



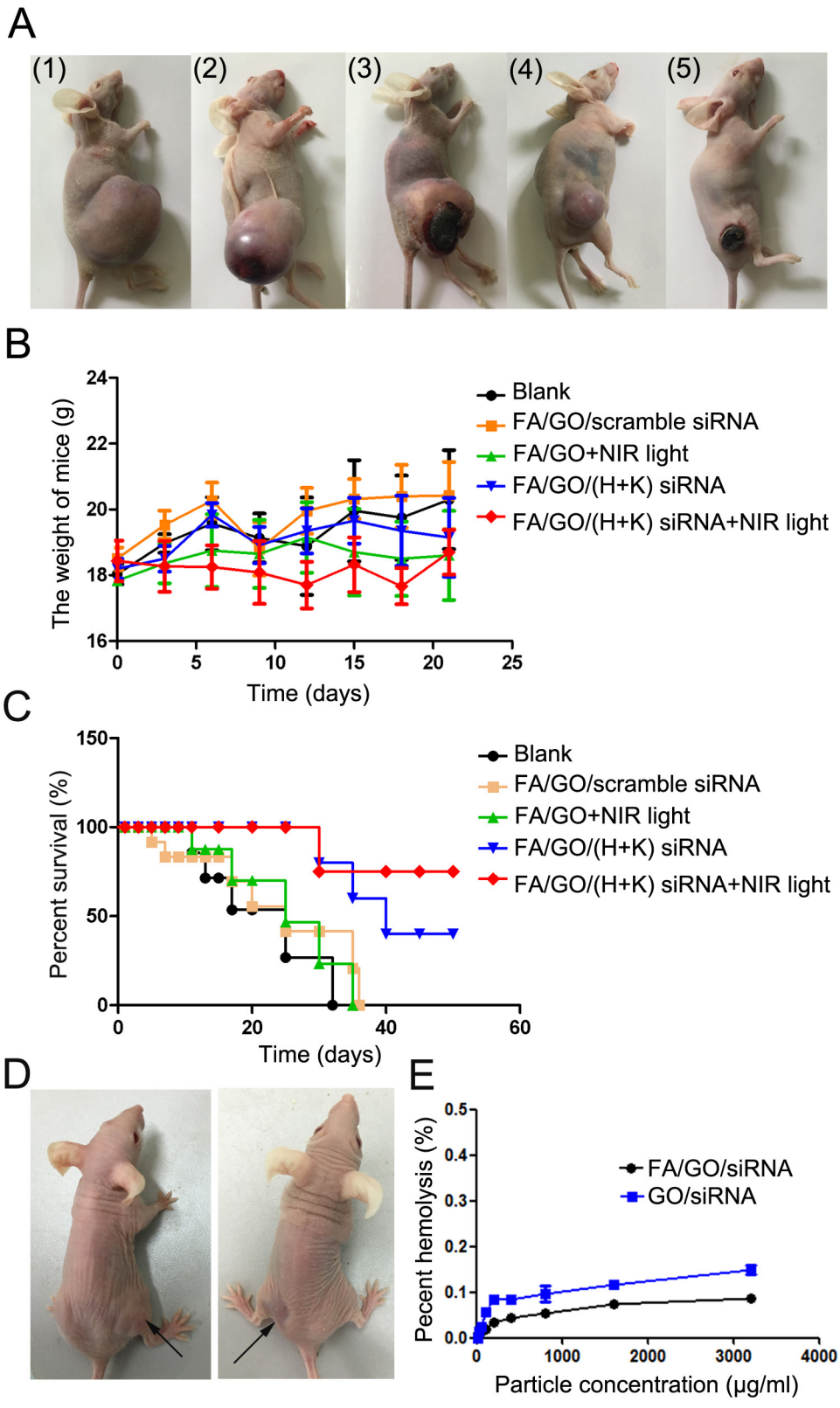
Supplementary Figure S5. Cell viability tests of different GO-basedformulations. The growth of QSG-7701 cells was not inhibited by FA/GO/siRNA nanoformulations. Phase contrast microscope images (A) and relative cell viabilities (B) of QSG7701 cells treated with PBS, FA/GO, FA/GO/scramble siRNA, FA/GO/K-Ras siRNA, FA/GO/HDAC1 siRNA and FA/GO/(H+K) siRNA for 4 hours, then all the cells were washed with PBS and re-incubated in fresh cell medium for designated time. Data were presented as the mean±SEM of triplicate experiments.



Supplementary Figure S6. 2D diagram to show the number of genes with change of expression level and gene ontology analysis in Miapaca-2 cells treated with different nanoformulations of PBS as blank, FA/GO/K-Ras siRNA, FA/GO/HDAC1 siRNA and FA/GO/(H+K) siRNA. Gene expression level and higher ranked 20 changed pathways were listed for Miapaca-2 cells treated with different nanoformulations (A) FA/GO/HDAC1 siRNA, (B) FA/GO/K-Ras siRNA or (C) FA/GO/(H+K) siRNA compared to blank compared to PBS as blank. The circle diameter represented gene number, the shades of blue represents the Q values.

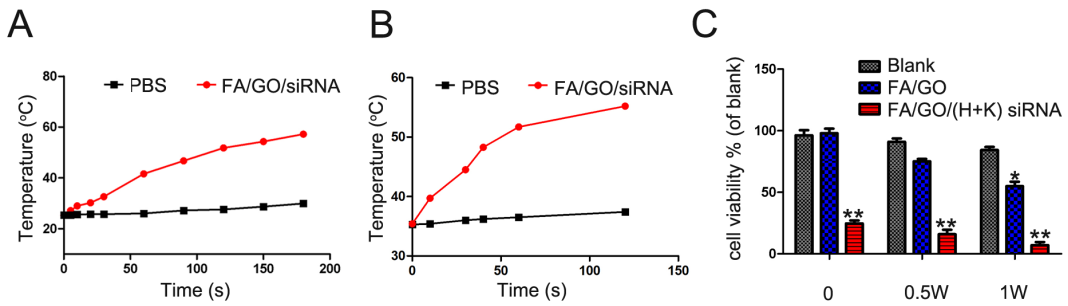


Supplementary Figure S7. The body distribution of GO/(H+K) siRNA complex (3), FA/GO/(H+K) siRNA complex (2) or PBS (1) at different time points after intravenous injections. (A) 0 hour, no optical signal was detected in the tumor site; the nanoformulations accumulated in the tumor site were then detected at 3 time points after incubation (B) 30mins, (C) 2hours, (D) 4hours. (E) The mice were sacrificed and the fluorescence of tumors and major organs were detected. The GO formulations (above) were confirmed to accumulate at the tumor, liver and kidney comparing with PBS (below).

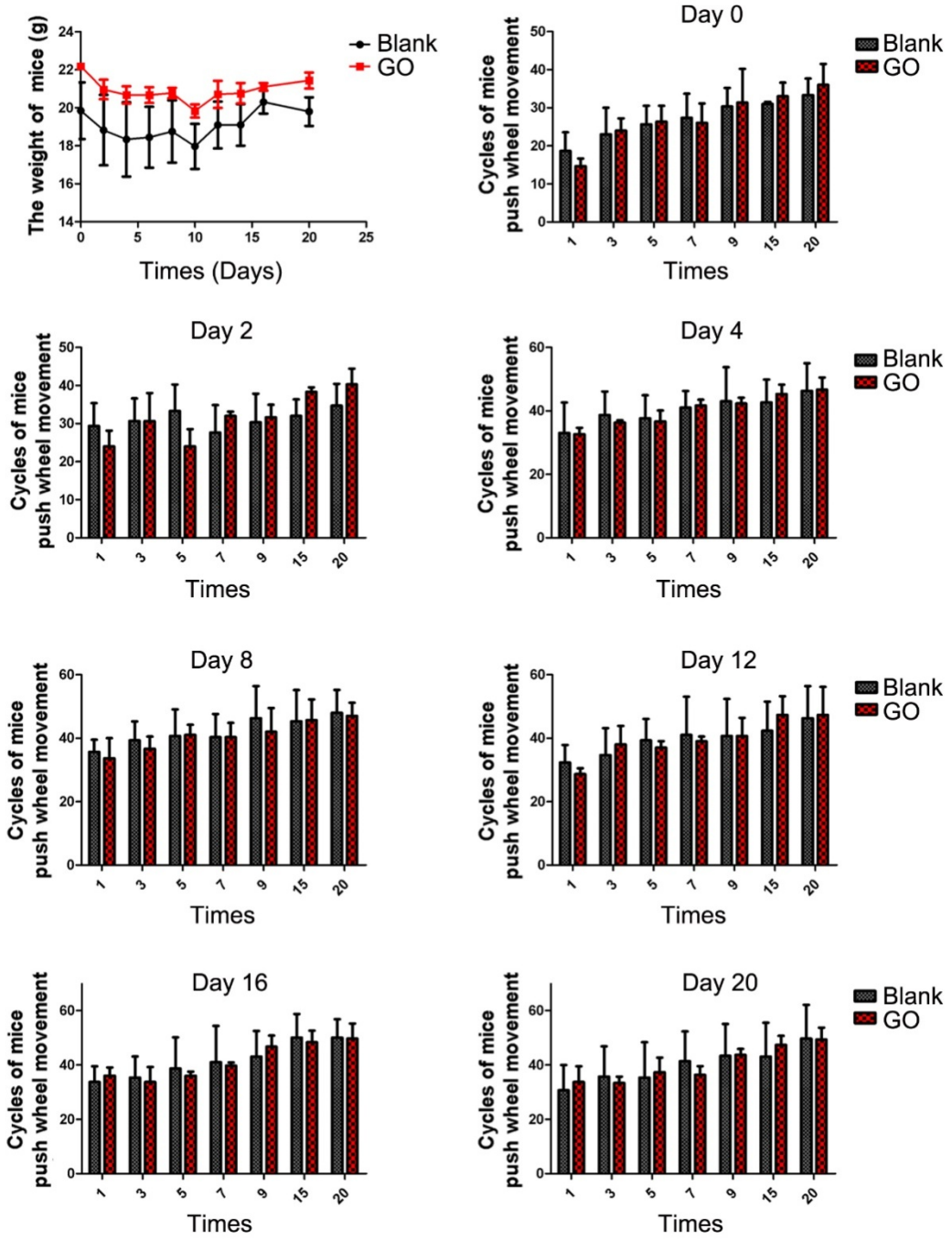


Supplementary Figure S8. Tumor inhibition effect of different GO nanoformulations. (A) Photograph at day 21 of representative mice. For each mouse, different GO

formulations were treated: (1) saline, (2) FA/GO/scramble siRNA, (3) FA/GO+NIR light, (4) FA/GO/(H+K) siRNA, (5) FA/GO/(H+K) siRNA+NIR light. (B) the weights of mice were detected. (C) Survival curves of mice treated by different GO nanoformulations as indicated. PBS as the blank. (D) Representative photograph of mice treated by the combination of GO-siRNA nanoformulations and NIR light for 60 days. The superior tumor inhibition ability of FA/GO/siRNA by intraperitoneal injection was observed although macrophages may take up some of the FA/GO/siRNA nanocomplex. (E) Percent hemolysis of RBCs incubated with different concentrations (12.5 to 3200 μg mL^{-1}) of GO/PEG/FA/SiRNA (black), GO/PEG/SiRNA(blue) for 3 h at 37° C with agitation. Data were presented as the mean±SEM of triplicate experiments.

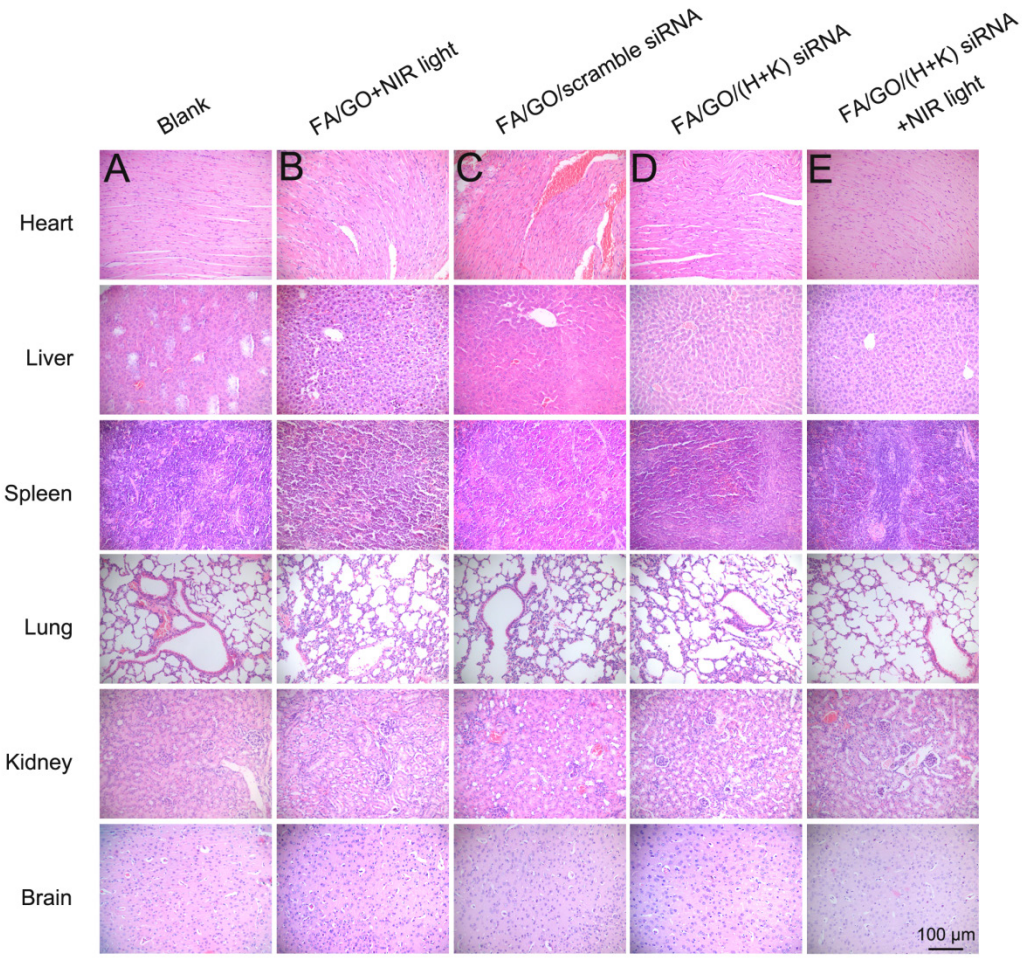


Supplementary Figure S9. Temperature dependence of FA/GO/siRNA nanocomplex under NIR light irradiation. FA/GO/siRNA nanocomplex were dispersed in PBS and the temperatures of the samples were measured by thermometer (T208, Digitron). (A) Within the first 3 minutes of the NIR light exposure in vitro, a 31 °C increment was observed for the FA/GO/siRNA nanocomplex (from 27 °C to 58 °C). On the other hand, only 5 °C increment was observed for PBS buffer solution (from 27°C to 32 °C). (B) Within the first 2 minutes of the NIR light exposure in tumors, a 19.8 °C increment was observed for the FA/GO/siRNA nanocomplex (from 35.6 °C to 55.4 °C). On the other hand, only 4.5 °C increment was observed for PBS buffer solution in tumors (from 35.4°C to 39.9 °C). (C) Cell viability tests of FA/GO/siRNA nanocomplex. Miapaca-2 cells were treated with PBS or FA/GO/(H+K) siRNA for 4 hours, then all the cells were exposed under NIR light with different power for 1 minute, and then re-incubated in fresh cell medium for designated time. Values are means ± SEM, n = 3; *, $P < 0.05$, **, $P < 0.01$ vs Control.

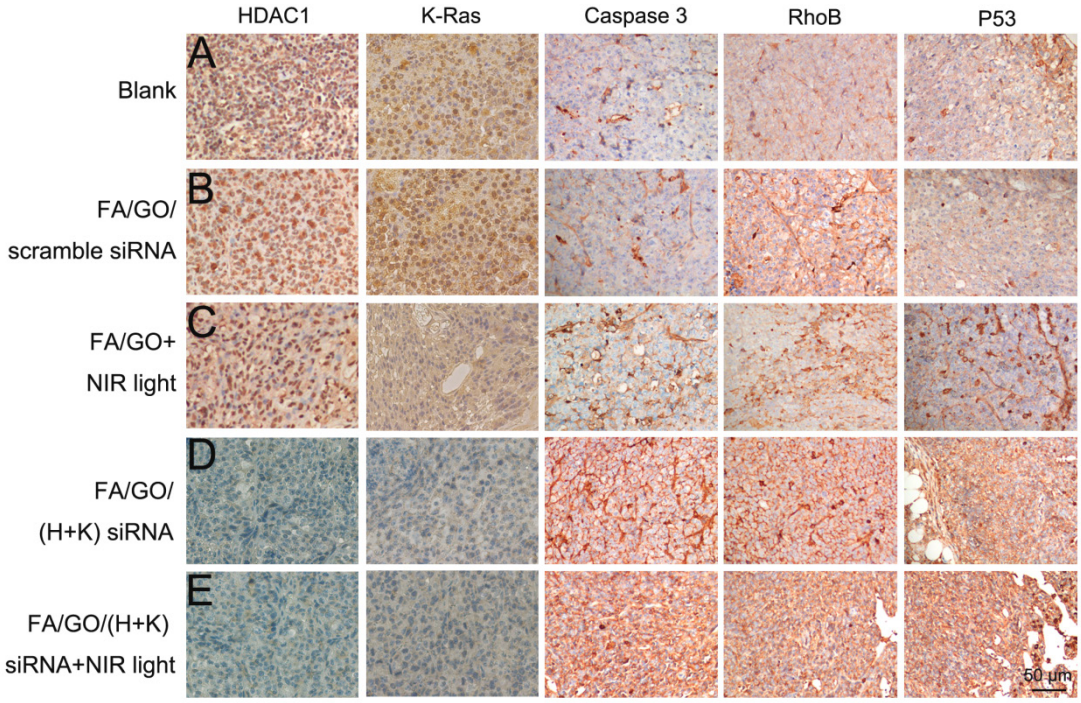


Supplementary Figure S10. The cage-wheel exercise assay was used to study the motor learning ability of mice. BALB/c mice were randomly divided into two groups and were subcutaneously injected with PBS or FA/GO (50 mg/kg). Over a period of 0 to 20 days, the weights of mice kept steady and the voluntary running cycles have been increasing steadily and exhibited no significant differences in these two groups, which indicated no obvious effect of motor learning ability of mice treated with FA/GO nanoformulations. The error bars represented the standard derivations (3 mice

per group).



Supplementary Figure S11. H&E stained tumor sections and organs collected from different groups of mice 3 weeks post treatment. (A) Blank, (B) FA/GO+NIR light (C) FA/GO/scramble siRNA (D) FA/GO/(H+K) siRNA (E) FA/GO/(H+K)siRNA+NIR light. MicroSpot Focusing Objective, 20X.



Supplementary Figure S12. Down-regulatingK-Ras and HDAC1 protein levels inhuman pancreatic carcinomas induced the high expression of P53, RhoB and Caspase-3 in the pancreatic carcinomas tissues collected from different groups of mice 3 weeks post treatment. (A) Blank, (B) FA/GO+NIR light (C) FA/GO/scramble siRNA (D) FA/GO/(H+K) siRNA (E) FA/GO/(H+K) siRNA+NIR light. HDAC1 and K-Ras proteins were high in normal pancreatic carcinomas tissues slides. The immunohistologic staining of human pancreatic carcinomas tissues were stained with anti-HDAC1, anti-KRas, anti-P53, anti-Caspase3 or anti-RhoB. MicroSpot Focusing Objective, 20X.

Supplementary Table S1 Zeta potential and hydrodynamic size of different GO-based nanoplex in PBS solution or DMEM medium.

In PBS solution:

Name of nanoplex	Grafted polymers	Zeta potential	hydrodynamic size
GO		-56.46 mV	678.6±8.34 nm
GO/PEG	PEG	-47.21 mV	412.7±5.18 nm
GO/PEG/PAH	PEG& PAH	+55.84 mV	503.5±7.67 nm
FA/GO/PEG/PAH	PEG& PAH	+54.13 mV	593.2±4.57 nm
GO/PEG/PAH/siRNA	PEG& PAH	+34.35 mV	567.7±7.73 nm
FA/GO/PEG/PAH/siRNA	PEG& PAH	+32.52 mV	637.9±4.65 nm

In DMEM medium:

Name of nanoplex	Grafted polymers	Zeta potential	hydrodynamic size
GO		-57.28 mV	625.4±5.29 nm
GO/PEG	PEG	-49.15 mV	385.9±3.17 nm
GO/PEG/PAH	PEG& PAH	+54.33 mV	463.5±7.67 nm
FA/GO/PEG/PAH	PEG& PAH	+50.16 mV	533.8±4.46 nm
GO/PEG/PAH/siRNA	PEG& PAH	+33.26 mV	507.2±4.82 nm
FA/GO/PEG/PAH/siRNA	PEG& PAH	+29.88mV	568.9±3.25 nm

Supplementary Table S2 The sequences of siRNAs

siRNA name	**Sequence (5'-3')**
Human-HDAC1siRNA	GCAAGCAGATGCAGAGATT
Human-Kras siRNA	GUUGGAGCUGAUGGCGUAG
sramble siRNA	CGAAGUGUGUGUGUGUGGC

Supplementary Table S3 Sequences of primers used for qPCR

Primer name	**Orientation**	**Sequence (5'-3')**
Human HDAC1	Forward	GGGATCGGTTAGGTTGCTTC
Human HDAC1	Reverse	TTGTCAGGGTCGTCTTCGTC
Human Kras	Forward	AGAGTGCCTTGACGATACAGC
Human Kras	Reverse	ACAAAGAAAGCCCTCCCCAGT
Human GAPDH	Forward	ACCACAGTCCATGCCATCAC
Human GAPDH	Reverse	TCCACCACCCTGTTGCTGTA
Human β-Actin	Forward	TCCAGCCTTCCTTCTTGGGTATG
Human β-Actin	Reverse	GAAGGTGGACAGTGAGGCCAGGAT
Human RhoB	Forward	GACGGCAAGCAGGTGGAGCT
Human RhoB	Reverse	ATGGGCACATTGGGACAGAA
Human BCL2	Forward	GACTTCGCCGAGATGTCCAG

Human BCL2	Reverse	GGTGCCGGTTCAGGTACTCA
Human Caspase-3	Forward	AGAACTGGACTGTGGCATTG
Human Caspase-3	Reverse	CTTGTCGGCATACTGTTTCA

Supplementary Table S4 Biodistribution data of the accumulation of particles

PBS group		**GO/siRNA treated group**		**FA/GO/siRNA treated group**	
Organ name	**Fluorescence accumulated data**	**Organ name**	**Fluorescence accumulated data**	**Organ name**	**Fluorescence accumulated data**
Brain	2.478 e+07	Brain	2.169 e+07	Brain	2.337 e+07
Heart	6.271 e+06	Heart	3.868 e+06	Heart	5.170 e+06
Liver	3.456 e+07	Liver	5.567 e+08	Liver	1.231 e+08
Spleen	1.908 e+07	Spleen	1.394 e+07	Spleen	2.221 e+07
Lung	2.155 e+07	Lung	1.956 e+07	Lung	1.499 e+07
Kidney	2.053 e+07	Kidney	5.237 e+07	Kidney	5.461 e+07
Tumor	2.795 e+07	Tumor	6.684 e+07	Tumor	1.842 e+08

Reference：

1. Liang YY, Li YG, Wang HL, Zhou JG, Wang J, Regier T, et al. Co3O4 nanocrystals on graphene as a synergistic catalyst for oxygen reduction reaction. Nat Mater. 2011; 10: 780-6.
2. Yin F, Yang C, Wang Q, Zeng S, Hu R, Lin G, et al. A Light-Driven Therapy of Pancreatic Adenocarcinoma Using Gold Nanorods-Based Nanocarriers for Co-Delivery of Doxorubicin and siRNA. Theranostics. 2015; 5: 818-33.
3. Yin F, Lan RF, Zhang XM, Zhu LY, Chen FF, Xu ZS, et al. LSD1 Regulates Pluripotency of Embryonic Stem/Carcinoma Cells through Histone Deacetylase 1-Mediated Deacetylation of Histone H4 at Lysine 16. Mol Cell Biol. 2014; 34: 158-79.
4. Yin F, Zhang BT, Zeng SW, Lin GM, Tian JL, Yang CB, et al. Folic acid-conjugated organically modified silica nanoparticles for enhanced targeted delivery in cancer cells and tumor in vivo. J Mater Chem B. 2015; 3: 6081-93.
5. Liao K-H, Lin Y-S, Macosko CW, Haynes CL. Cytotoxicity of Graphene Oxide and Graphene in Human Erythrocytes and Skin Fibroblasts. ACS Appl Mater Interfaces. 2011; 3: 2607-15.
6. Yang G, Lai CS, Cichon J, Ma L, Li W, Gan WB. Sleep promotes branch-specific formation of dendritic spines after learning. Science. 2014; 344: 1173-8.