Electronic Supplementary Material (ESI) for Nanoscale Advances
This journal is © The Royal Society of Chemistry 2021

Electronic Supplementary Information (ESI)

Synthesis of Nanodrug Using Metal-Based Nanozymes Conjugated with Ginsenoside Rg3 for Pancreatic Cancer Therapy

Xiaoxiong Zhao,[#a, b] Jicheng Wu,[#c, d] Kaixin Zhang,[c, d] Danjing Guo,[e] Liangjie Hong,[e] Xinhua Chen,[b, e] Ben Wang,*[c, d] and Yujun Song*[a, b]

[a]Center for Modern Physics Technology, School of Mathematics and Physics, University of Science and Technology Beijing, Beijing 100083, China.

[b]Zhejiang Key Laboratory for Pulsed Power Technology Translational Medicine, Hangzhou 310000, China.

[c]Cancer Institute (Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education), The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China.

[d]Institute of Translational Medicine, Zhejiang University, Hangzhou 310029, China.

[e]Key Laboratory of Combined Multi-Organ Transplantation, Ministry of Public Health, Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China.

Experimental

Synthesis of $Fe@Fe_3O_4$ Nanoparticles

$Fe@Fe_3O_4$ NPs were synthesized using a simple programmed microfluidic strategy. Polyvinylpyrrolidone (PVP) and $FeCl_2 \cdot 4H_2O$ were dissolved into ultrapure water to form the metal salt solution. $NaBH_4$ was dissolved into N-methyl-2-pyrrolidone (NMP) to form the reducing solution. Then, metal salt solution and reducing solution are sucked into each of the syringes and fixed in the syringe pump platform. The metal–salt solution and the reducing solution are preheated to 90°C in the thermostatic tank by the microchannel. At a flow rate of 1.0mL/min per pump, initiating the reducing reaction and the nucleation. The obtained fresh NPs solution was collected at 0°C in the product collector controlled in the thermostatic tank. Then perform centrifugation, washing, and drying for future use.

The process of $Fe@Fe_3O_4$ NPs coupled with Rg3

First, anhydrous toluene solution which contains five mass% of (3-aminopropyl) trimethoxysilane (APTMS) was added into the NPs solution. Then the mixed solution is stirred for 24 hours at room temperature. Then perform centrifugation, washing, and redispersing into dimethyl sulfoxide (DMSO). The bifunctional amine active cross-linker of DSS was subsequently added. The obtained mixture is incubated and mixed for more than two hours at room temperature and then centrifuged, washed, and redispersed into dimethyl sulfoxide (DMSO). Similar to the surface amination of NPs by APTMS, obtain the aminosilane-modified ginsenoside Rg3. Then, the aminosilane-activated ginsenoside Rg3 was dissolved into the DSS activated NPs are added into the solution. Then the final product is centrifuged and washed with water for two times. The washed slurry is dried and kept for future use.

Characterization of Nanoparticles and RNME

Sizes, shapes, and core-shell morphology of NPs were characterized by transmission electronic microscopy (TEM, JEM-3010, 300 kV, dot resolution: 0.17 nm). Their crystal phase was characterized by X-ray diffraction (XRD, using the copper Kα wavelength (λ= 1.540 56, Kα line, RINT2000) at a scanning rate of 5°/min. The X-ray photoelectron spectroscopy (XPS) was used to determine the elemental composition and chemical and electronic state of the related elements in NPs by detecting their thin films. Fourier Transform infrared spectroscopy (FT-IR, Nicollet IMPACT 400D, Nicollet Inc.) was used to characterize the composition changes of the samples.

Ginsenoside Rg3 coupling efficiency

Ginsenoside Rg3 coupling efficiency was determined by high-performance liquid chromatography (HPLC). Ginsenoside Rg3, which failed to be coupled, was still dissolved in the solvent and could not be centrifuged. Therefore, after the coupling is completed, the centrifuged supernatant is subjected to an HPLC test, which confirms the concentration of ginsenoside Rg3 remaining in the supernatant. It was found that the coupling efficiency remained basically unchanged when the ratio of nanoparticles to ginsenoside Rg3 was 1:0.25 to 1:1.

Still, when the ratio of ginseng was increased, the coupling efficiency decreased significantly. Therefore, we confirm that the best coupling mass ratio is 1:1.

Release efficiency of RNME

In the RNME release experiment, two methods are used to verify. First, HPLC measures the release of Rg3 to calibrate the release amount, and performs HPLC detection at different times under physiological conditions and the tumor microenvironment. The final release rate is the ratio of the active area of releasing Rg3 to the total area of ginsenoside Rg3 participating in the reaction.

Secondly, ICP measures the release of iron to calibrate the release amount, and RNME is dissolved in 10mL PBS. Then collect a 200 μl sample of the supernatant at the ideal time point, while replenishing the solvent. The ICP-MS test of Fe was carried out, and the same release experiment was performed to simulate the tumor microenvironment in vitro.

Intracellular uptake of Fe.

ICP-MS was used to measure the uptake of Fe in the cells, and L3.6pl cells were seeded in a petri dish overnight at a density of 6×10^6 cells. These cells were then treated with different drugs and incubated at 37°C for 24 h. After washing 3 times with PBS, 3×10^6 cells were selected, and the cells were lysed with cell lysis buffer to test the Fe content in ICP-MS.

Free radicals of •OH and $O_2^{\bullet-}$ detection and dissolved oxygen analysis

5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) spin-trapping adduct was used to detect •OH and $O_2^{\bullet-}$ generation by electron spin resonance spectroscopy (Bruker A300 EPR Spectrometer, microwave frequency=9.84 GHz, microwave power=20.43 mW). The specific experimental program is as follows:10 μL of RNME or NPs with a concentration of 200 μg mL^{-1}, 10 μL of H_2O_2, 50 μL of DMPO, 20 μL of NaAc buffer and were added into 160 μL of DMSO solution for $O_2^{\bullet-}$ detection. 10 μL of RNME or NPs with a concentration of 200 μg mL^{-1}, 10 μL of H_2O_2, 50 μL of DMPO were added into 180 μL of NaAc buffer for •OH detection. All the samples were reacted for 2 min. Oxygen electrode on Multi-Parameter Analyzer (JPSJ-606L, Leici China) was employed to detect the O_2 generation.

Co-staining picture of cells by CLSM

Co-staining of cells with Calcein-AM and PI: Inoculate the cells in a special culture plate for CLSM and incubate for 24 hours. RNME (200 μg mL^{-1}) was incubated with the cells for another 24 hours, and then washed twice with PBS, and then after 30 minutes of incubation with calcein-AM and PI, the cells were washed twice with PBS. CLSM observes green and red fluorescence. Co-staining of cells with Hoechst and DCFH-DA: Cell culture was the same as above. After various treatments, 1 mL of DCFH-DA was incubated for 30 minutes, then washed 3 times, and then incubated with Hoechst for 20 minutes and washed twice. Blue and green fluorescence were observed by CLSM. Stain cells with C11-BODIPY and JC-1: cell culture was the same as above. After various

treatments, 0.5 mL C11-BODIPY or JC-1 was incubated for 30 minutes and then washed 3 times. Green fluorescence or red and green fluorescence were observed with CLSM.

Cell culture

Pancreatic cancer cell line L3.6pl was purchased from ATCC and maintained in alfa-MEM medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Cell viability assay and Flow cytometry

The cell counting kit 8 (CCK-8; Dojindo, Kumamoto, Japan) was used to define the number of viable cells in a 96-well plate by measuring the absorbance at 450 nm. Cells were seeded in a 12-well plate at a concentration of 5×10^5 and incubated for 24 hours. Add RNME (200 μg mL^{-1}) and incubate for 24 hours. The cells were then washed twice and incubated with DCFH-DA for 30 minutes. The cells were collected and washed for further analysis by flow cytometry.

Apoptosis detection and transwell assays

The cells were seeded in a 6-well plate and then incubated with RNME (200 μg mL^{-1}) for 24 hours. The cells were harvested by trypsin, washed twice, and stained with FITC-labeled Annexin V for 30 minutes and stained with phosphatidylserine. Measure apoptotic cells in chemistry. The cells were then stained with propidium iodide (PI) and incubated for 10 minutes in the dark at room temperature. Measure Annexin V-FITC/PI-labeled cells on a flow cytometer.

For migration and invasion assays, the cells are digested, then resuspended in alfa-MEM, and then seeded into the upper chamber, which contains a Matrigel 1 coating (invasion) or not (migration). Place alfa-MEM (600 μl) containing 10% fetal bovine serum as a chemoattractant in the lower chamber. After treating the cells with different drugs (200 μg mL^{-1}) at 37°C for 24 hours, wipe the cells on the upper surface of the chamber with a cotton swab, remove the cells on the lower surface of the membrane and fix it with 4% formaldehyde in PBS, and use 0.01% crystals Violet stained and counted under an optical microscope. The experiment was carried out three times.

Western blotting analysis.

Denatured protein was separated on an SDS-polyacrylamide gel and transferred to the Hybond membrane, which was then blocked overnight in 5% skim milk in TBST. For immunoblotting, the membrane was incubated for 120 min with the primary antibody. Then, it was rinsed by TBST and incubated with IgG conjugated to horseradish peroxidase for 60 min. Bands were visualized with Bio-Rad ChemiDoc Touch by BeyoECL-Plus detection reagents. GPX4 _Abcam_#ab125066

Terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (TUNEL).

A terminal transferase dUTP nick end labeling (TUNEL) detection kit was used to identify apoptotic cells. By incubating the sample with freshly prepared 3% H_2O_2 at room temperature for 10 minutes, washing 3 times, and blocking the endogenous peroxidase activity. The sample was incubated with proteinase K working solution for 15 minutes and washed 3 times. The tissue and TUNEL reaction mixture were then incubated at 37°C for 1 hour. Add DAPI solution to the tissue and incubate for 2 minutes. After washing 3 times, fix the tissue with anti-fading fixative. Then, view the sample through CLSM.

Histology and immunohistochemistry

The tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Use hematoxylin and eosin (H&E) to stain the sections or for immunohistochemical research. For immunohistochemistry, the sections were deparaffinized, rehydrated, repaired, stained with the primary antibody for 24 h, and reacted with the horseradish peroxidase-conjugated secondary antibody for 1 h, and then used diaminobenzidine as the antibody. The substrate produces an observable brown color. Use primary antibodies: rabbit anti-HIF1-α, mouse anti-Ki67, and rabbit anti-VEGF.

Animals

Animals Order 4-6 weeks old male nude mice and observe for one week to adapt to the laboratory environment. Animal research was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhejiang University. The animal study was conducted by a licensed doctor from the Department of Hepatobiliary and Pancreatic Surgery of the First Affiliated Hospital of Zhejiang University.

In vivo assay

For mice adapted to the environment, we prepared L3.6pl pancreatic cancer cells at a dose of 25 μL each and had 1 million cells. First, the mice are anesthetized. After anesthesia, the surgical injection was performed. The left lateral position of the mouse was inverted and fixed. A 1.0 cm incision was made 0.5-1.0 cm below the bottom rib to expose the abdominal cavity to extract the spleen. Pull the tail of the pancreas according to the spleen and insert the prepared pancreatic cancer cells into the pancreas. After injection, press the injection site with a sterile cotton swab to prevent cells from penetrating the abdominal cavity. After the cells were inoculated, the tumor formation and tumor size of the mice were detected by a fluorescence imaging system to determine the administration time. The mice were then randomly divided into groups and given different drugs every two days at a concentration of 20 mg/kg.

Pharmacokinetics

Pharmacokinetic analysis, the mice were randomly divided into groups (n = 3) and injected with different drugs. After the injection, a blood sample (0.1 mL) was taken from a capillary tube inserted into the eye socket at the required time, and anticoagulant was added for centrifugation. HPLC and ICP-MS were used

to measure the concentration of Rg3 and Fe in the serum.

Biodistribution

Biodistribution analysis, the main organs and tumor tissues were collected. Then the samples were lyophilized, weighed, and digested using concentrated nitric acid. The amount of iron was analyzed by ICP-MS.

Statistical analysis.

All cell experiments were independently repeated at least 3 times, and the data were expressed as the mean plus or minus the standard deviation. Statistical tests for data analysis include chi-square test and 2-tail t-test. P values of 0.05 or less were considered statistically significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The statistical analyses were performed using the SPSS 17.0 statistical software package.

Figures and Tables

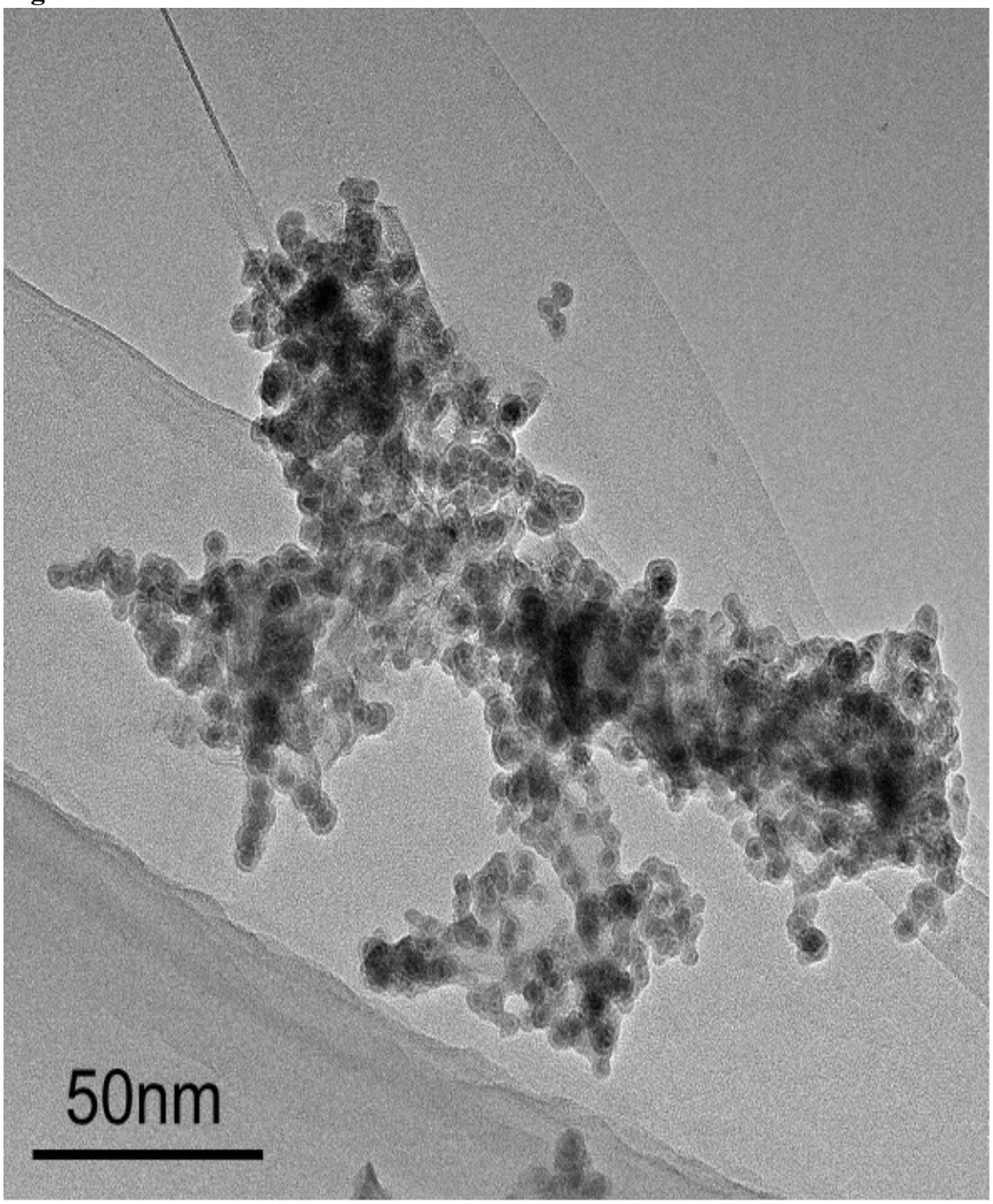

Fig S1. Wide-view TEM images of $Fe@Fe_3O_4$ NPs.

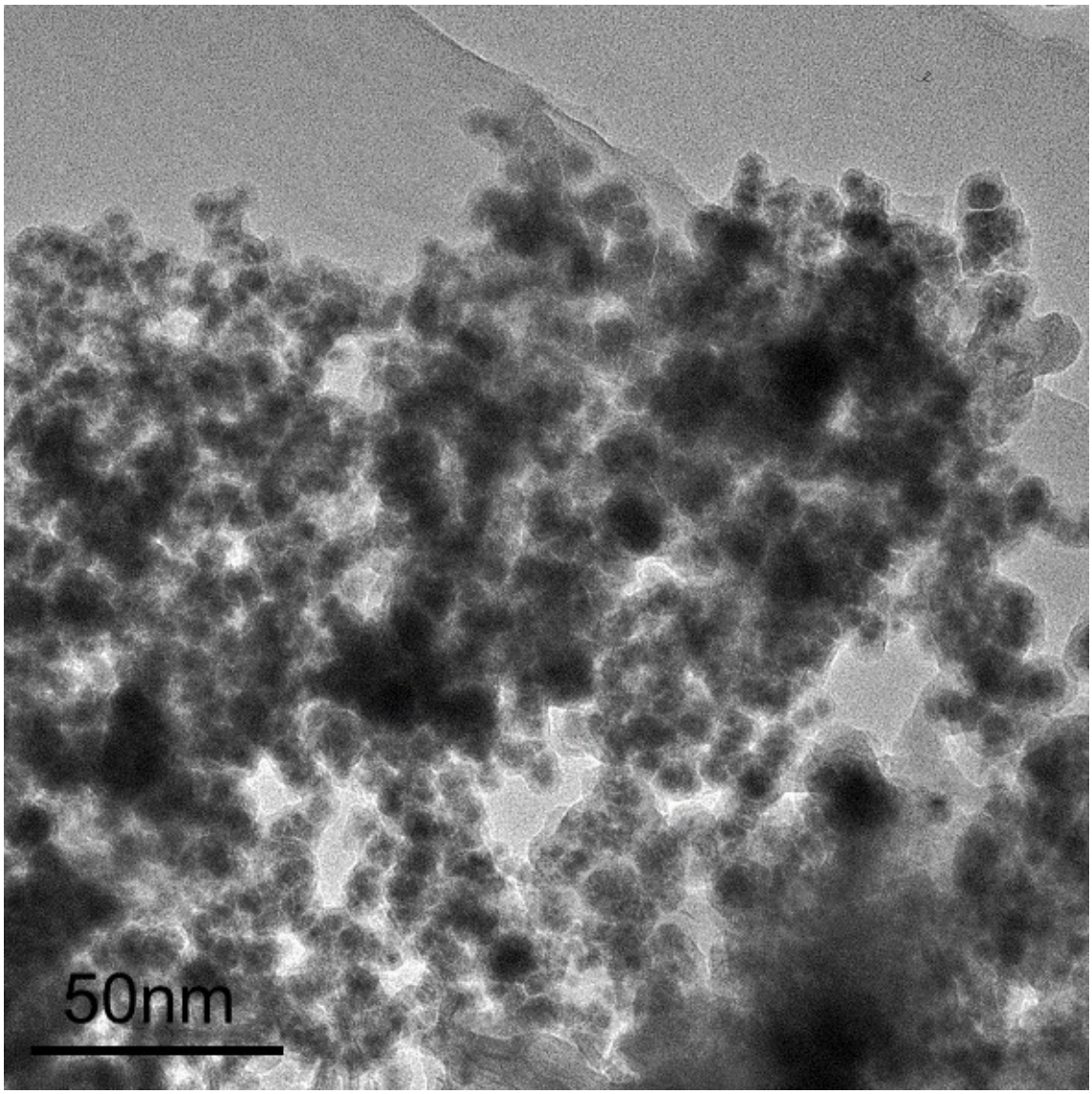

Fig S2. Wide-view TEM images of RNME.

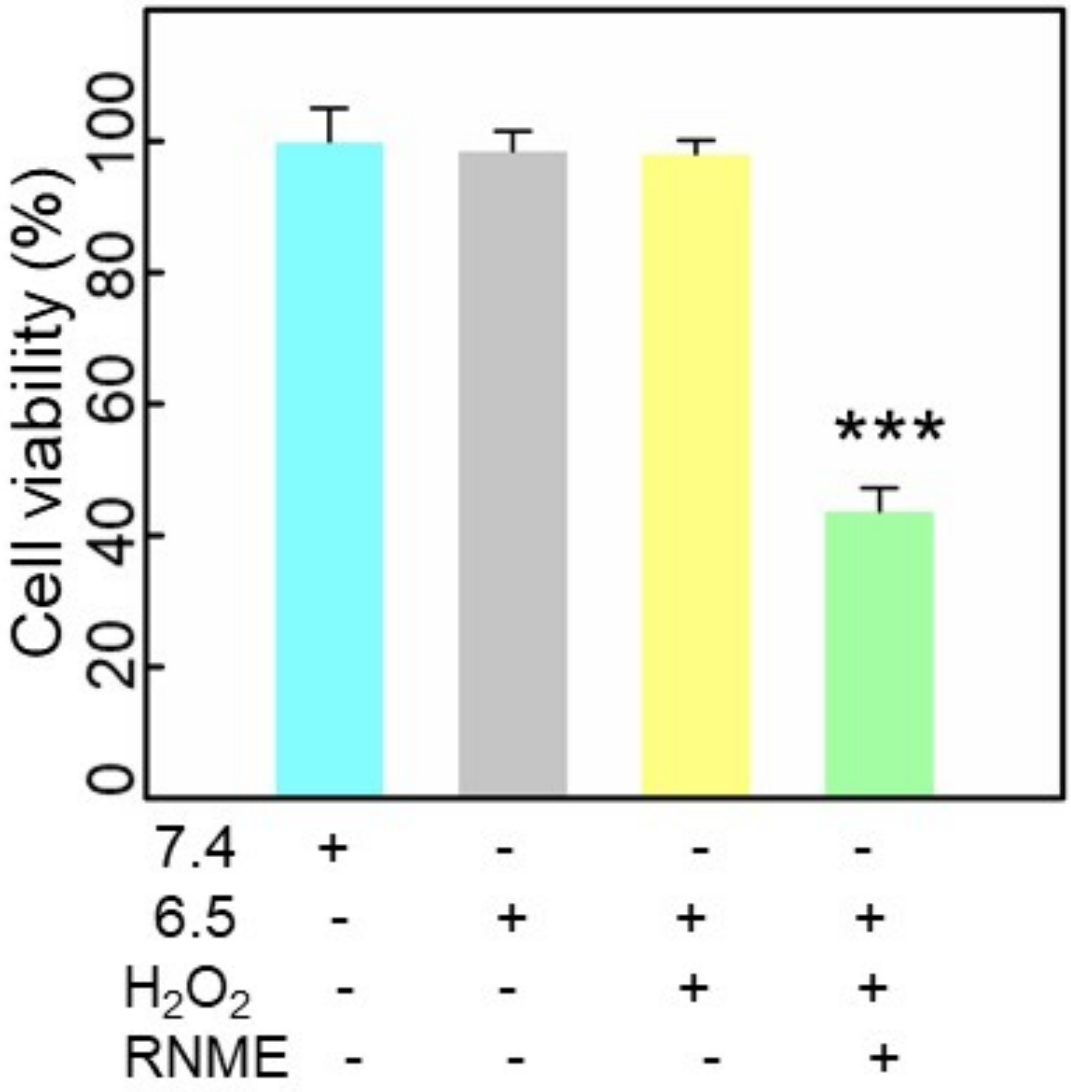

Fig S3. Cell viability of mimics tumor microenvironment in vitro.

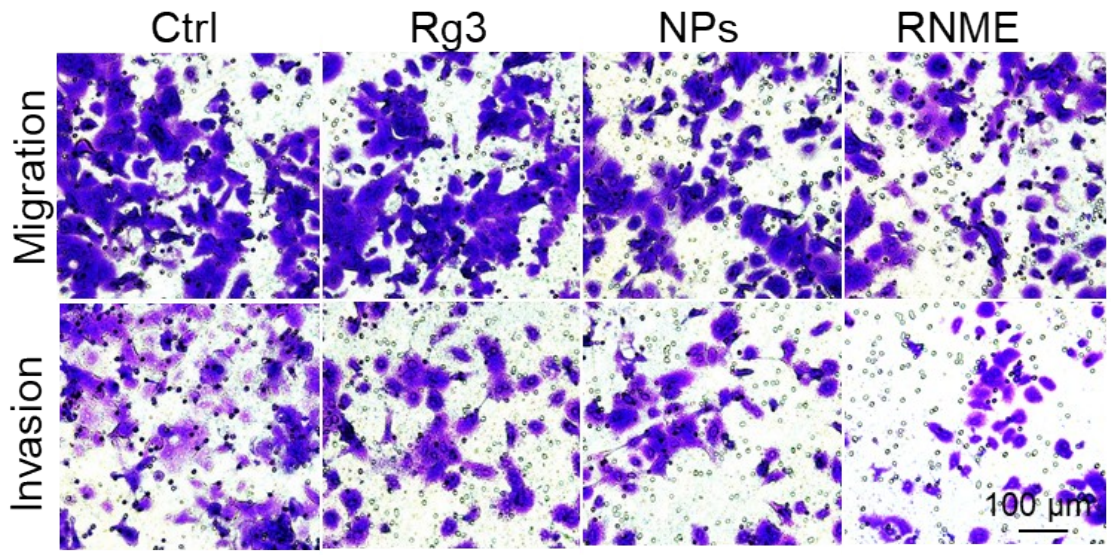

Fig S4. Cell migration and invasion ability of L3.6pl cells treated with different drugs for 24 hours.

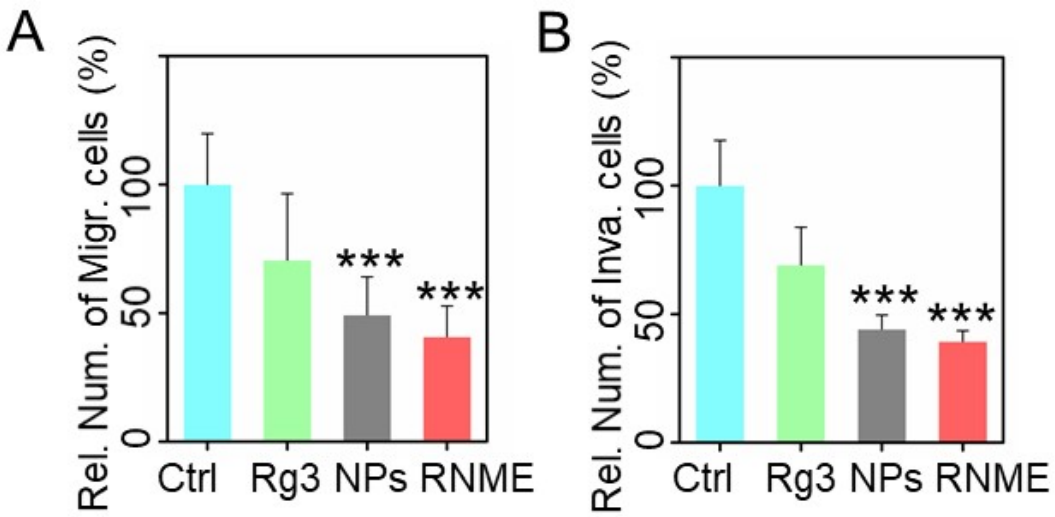

Fig S5. Quantify cell migration (A) and invasion (B) after treatment by different drugs.

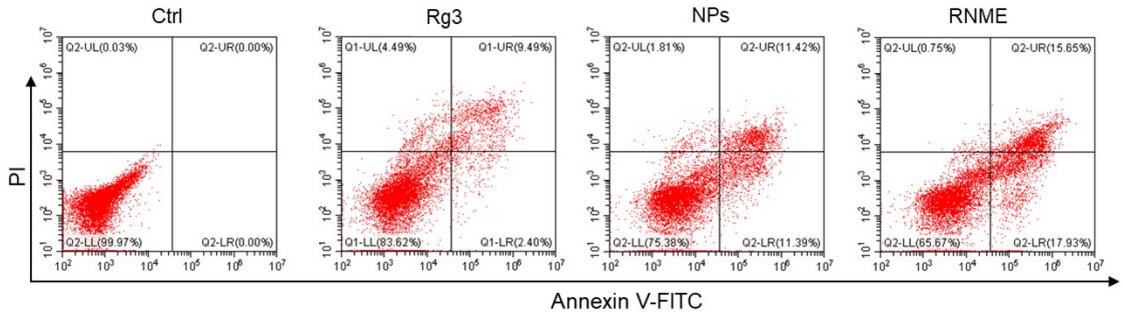

Fig S6. The annexin V-FITC/PI apoptosis detection analysis of L3.6pl cells treatment with different drugs after 24 h.

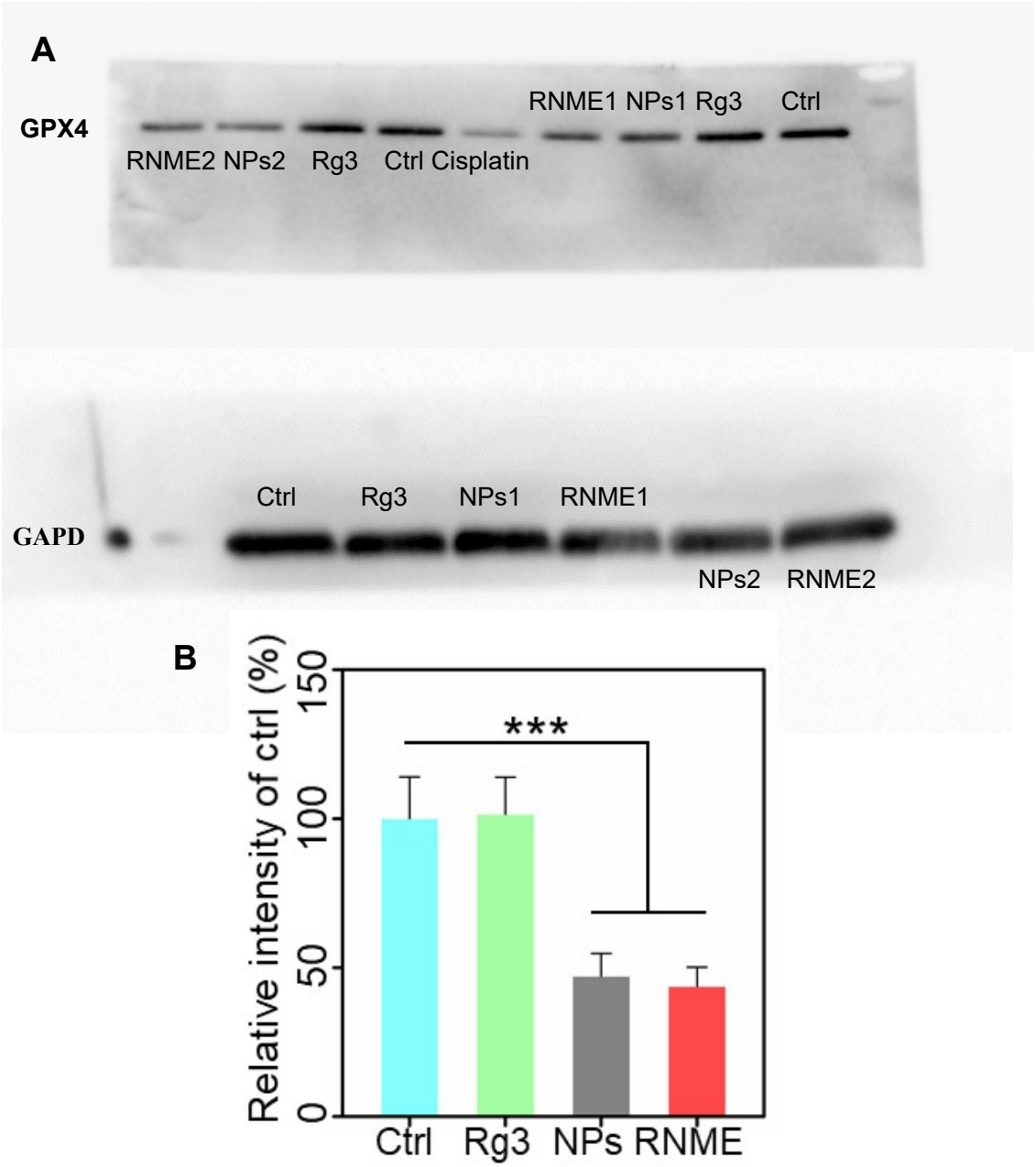

Fig S7. (A) GPX4 (glutathione peroxidase 4) protein levels were determined by Western blot analysis after incubation with different drugs for 24 hours (Note: RNME1 and RNME2 are the same naomedicine, RNME; NPs1 and NPs2 are the same nanoparticles, NPs; GAPD: glyceraldehyde-3-phosphate-dehydrogenase). (B) Western blot quantitative analysis of GPX4.

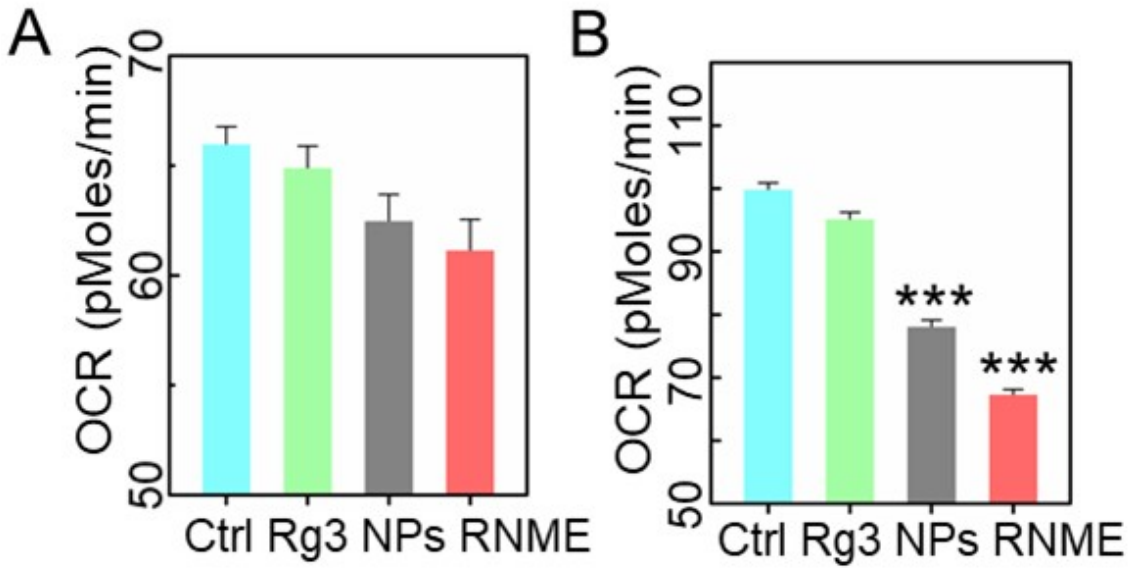

Fig S8. Quantitative analysis by normalizing OCR levels for the cell showed the basal respiration rate (A) and the maximum respiration rate (B) by different drug treatments.

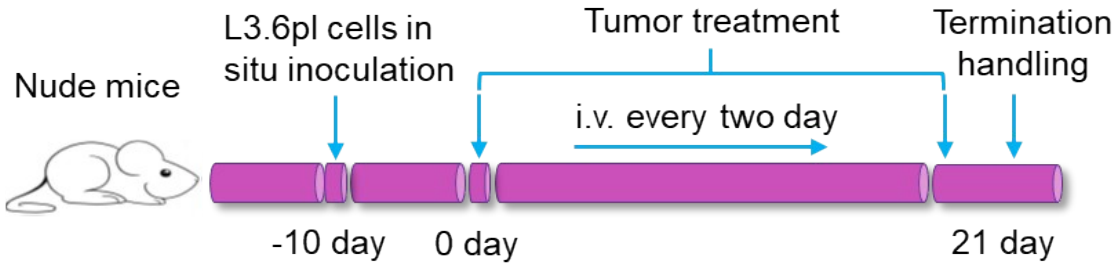

Fig S9. The procedure of animal experiments.

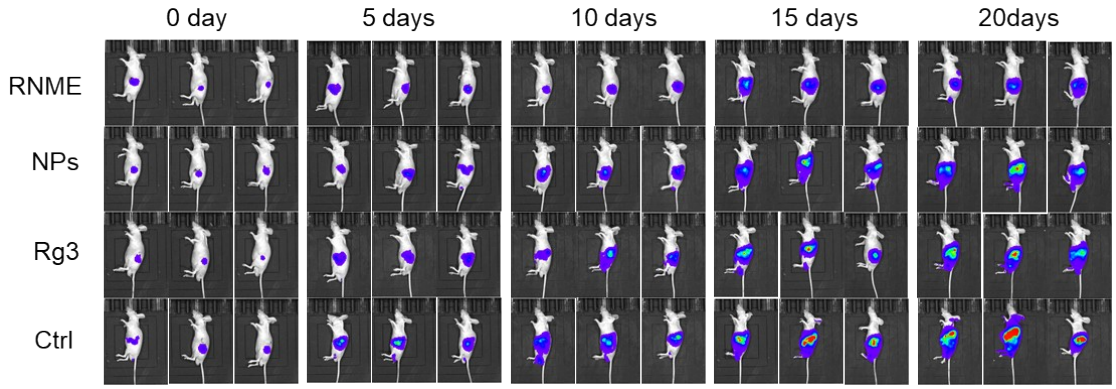

Fig S10. Bioluminescence imaging of the tumor at dynamic times after different drug treatments.

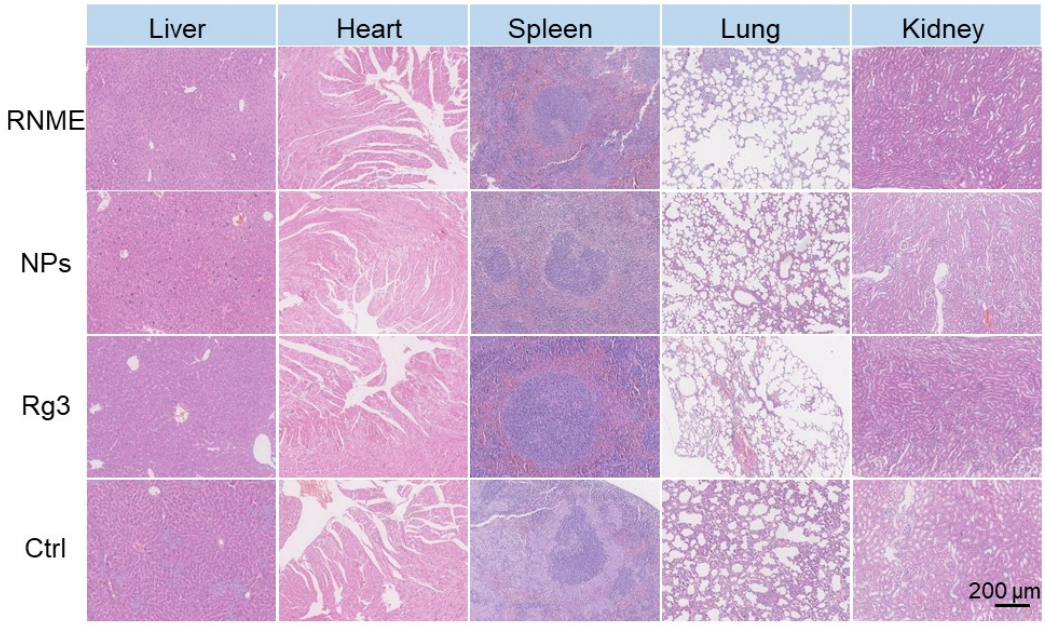

Fig S11. H&E staining assay of major organs of mouse treated by different drugs.

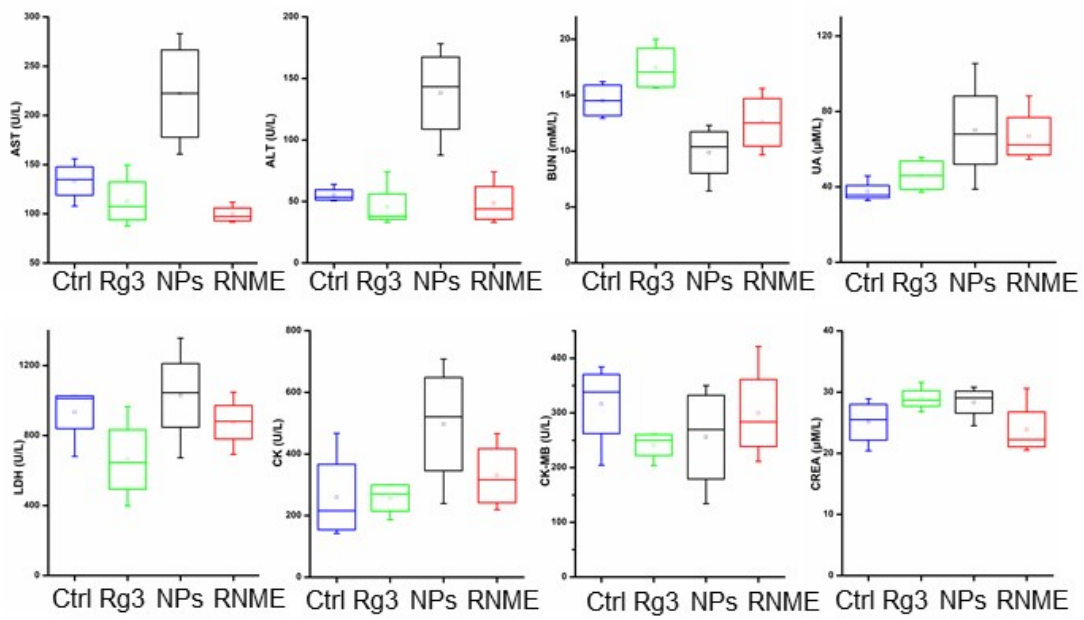

Fig S12. Blood biochemistry tests of the mouse were treated with different drugs.

Table S1. Elemental quantitative analysis by XPS.

Sample / Element	**NPs**	**NPs -APTMS**	**NPs -APTMS -DSS**	**RNME**
Fe 2p (%)	2.34	2.61	2.16	1.36
C 1s (%)	68.12	67.75	73.75	72.84
N 1s (%)	0.88	3.02	2.23	2.07
B 1s (%)	8.01	4.45	2.44	2.75
O 1s (%)	20.64	18.65	16.52	18.75
Si 2p (%)	0	3.52	2.89	2.23