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Supporting Information

Amorphous ferric oxide-coating selenium core-shell nanoparticles: A self-preservation Pt(IV) platform for multi-modal cancer therapies through hydrogen peroxide depletion-mediated anti-angiogenesis, apoptosis and ferroptosis

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Methods

Materials. All chemical reagents were used as received without further purification. Sodium selenite, 3′, 3′, 5′, 5′-tetramethylbenzidine (TMB), glutathione (GSH), N-ethyl-N′-(3-(dimethylamino)propyl) carbodiimide (EDC), and polyethyleneimine (PEI) were purchased from Aladdin Chemical Co. Ltd. (Shanghai, China). $FeCl_2 \cdot 4H_2O$, vitamin C, N-hydroxysulfosuccinimide, and $(NH_4)_2SO_3$ were provided by the Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The H_2O_2 ELISA Kit were purchased by Shang Hai Jianglai Biological Technology Co., Ltd. The cell counting kit-8 (CCK-8) assay was obtained from Dojindo (Japan). iRGD-PEG-NH_2 was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. The 2,7-dichlorofluorescein diacetate (DCFH-DA) was obtained from Nanjing Jiancheng Bioengineering Institute. Rabbit monoclonal anti-PARP (#9542), anti-cleaved PARP (#5625), anti-Bax (#5023), anti-cleaved Caspase 9 (#52873), anti-Caspase 7 (#12827), anti-cleaved Caspase 7 (#8438), anti-Ki67 (#9129), anti-Caspase 3 (#14220) and anti-cleaved Caspase 3 (#9664), mouse monoclonal anti-Caspase 9 (#9508) antibodies were purchased from CST. Mouse monoclonal antibodies against Bcl-2 (sc-7382) and GPX-4 (sc-166570) were obtained from Santa Cruze. Mouse monoclonal antibodies against β-tubulin (T0023) was provided by Affinity Antibody. ROS inhibitor N-Acetyl-cysteine (YZ-140671) and endocytosis inhibitor AmilorideHCldehydrate (A0080) were purchased from Solarbio Life Science.

Synthesis of Nano-selenium. Firstly, NSe could be fabricated through a classical reduction method. Briefly, the Vc (20 mg) and PEI (1.0 g) were uniformly dissolved into 50 mL of the distilled water. Then, under vigorous stirring conditions, the solution of sodium selenite (5mg/mL) was added into the mixed solution and continuously stirred for 6 hours. After that, the red solution was collected and centrifuged under 21000 rpm/min for 20 min. Finally, the product (NSe) was collected for later use.

The synthesis of NSe-Pt. The platinum(IV) prodrug was synthesized through previously reported [1]. 50 mg of NSe was dispersed into 30 mL of DMSO solution including platinum prodrug (2 mg/mL). Subsequently, the EDC (100 mg) and NHS (100 mg) were quickly added into the resulting solution under stirring conditions overnight. Afterwards, the product (NSe-Pt) was collected through centrifugation with 21000 rpm/min.

The Fabrication of iAIO@NSe Nanoplatform. The prepared NSe-Pt (50 mg) were dispersed

into 20 mL of the mixed solution including $FeCl_2$ (2.5 mg/mL) and $(NH_4)_2SO_3$ (2.5 mg/mL) under magnetic stirring at 500 rpm/min. Subsequently, the concentrated ammonium hydroxide (1 mL) was quickly injected into the resulting solution and the the solution was continuously stirred for 1 hour. After that, the solution was treated using a magnet to remove magnetic nanoparticles. The product (AIO@NSe-Pt) was sonicated and collected through step-by-step centrifugation.

In order to excellent tumor-targeting and biocompatibility, the iRGD-PEG-COOH was further conjugated to the surface of AIO@NSe-Pt through amide bond. Firstly, 50 mg of iRGD-PEG-COOH was dissolved into 20 mL of DMSO solution. And then the EDC (50 mg) and NHS (50 mg) were added to the resulting solution and continuously stirred for 30 min. After that, 50 mg of AIO@NSe-Pt was uniformly dispersed into the mixed DMSO, and stirred for overnight. Afterwards, the product was centrifuged, washed, and dried using freeze drying.

Release Behavior of Fe from iAIO@NSe-Pt. The iAIO@NSe-Pt (5 mg) was uniformly dispersed into PBS (10 mL) with different pH conditions (7.4, 6.5, and 5.5), respectively. Then, the mixed solutions were incubated in a shaker for 24 h at room temperature. After that, the solution was centrifuged at 14000 rpm at different time intervals, and the supernatant liquid was collected. Finally, Fe concentrations in the supernatant liquid were measured using inductively coupled plasma optical emission spectrometer (ICP-OES).

Release Behavior of Pt from iAIO@NSe-Pt. The iAIO@NSe-Pt (5 mg) was uniformly dispersed into phosphate-buffered saline (PBS) (10 mL) with different pH conditions (7.4, 6.5, and 5.5) in the absence and presence of GSH (10 mM), respectively. Then, the mixed solutions were shook at 150 rpm/min for 24 h. At different time intervals, the mixtures were centrifuged at 14000 rpm/min. Afterwards, the supernatant liquid was collected and Pt concentrations in the supernatant liquid were measured using ICP-OES.

Fenton-Catalytic Experiment. TMB color test was used to assess the Fenton catalytic performance of iAIO@NSe-Pt. Briefly, the iAIO@NSe-Pt with different concentrations were added into the mixed solution containing PBS (pH 4.5, 5.0, 6.0, 6.5, 7.4, 1.5 mL), TMB (1 mg/mL, 200 μL) and H_2O_2 (30 μL, 30%). After 5 min, the absorbance of the solutions were detected via UV-Vis spectrometer at the wavelength of 650 nm. In addition, the ·OH production was also monitored by ESR spectroscopy. In brief, the iAIO@NSe-Pt and USIO (

pH 4.5, 1 mL, 20 mM) were uniformly dispersed into PBS with and without H_2O_2 (10 mM). Subsequently, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 100 μL, 200 mM) were used to capture ·OH. After treatment for 20s, these samples were measured by ESR spectroscopy.

Cell Culture. Human breast cancer cell MDA-MB-231 was cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) containing 10% fetal bovine serum (Hyclone) and penicillin-streptomycin (100 U/ml penicillin and 0.1 mg/ml streptomycin). Mouse breast cancer cell 4T1 was cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.All cell cultures were incubated at 37°C in the presence of 5%CO_2. HUEVC cells were purchased from Shanghai Zishi Biotech Co., Ltd. and cultured in ECM medium supplemented with 5% FBS (Gibco), 1% ECGF and 1% penicillin-streptomycin in 5% CO_2 atmosphere at 37 °C.

Cell Viability Assay. MDA-MB-231 and 4T1 cells were digested with 2.5% trypsin (Solarbio) and collected in tubes, and then the cell number were counted by Countess™ II Automated Cell Counter (AMQAX1000, ThermoFisher) as manufacturer's instruction. The cells were seeded into 96-well plates and then incubated with free Pt(IV), AIO@NSe-Pt, iAIO@NSe-Pt, and iAIO@NSe-Pt+NAC for 24 h and 48 h. Subsequently, the culture media was removed and the corresponding cells were washed with PBS for three times. The complete cell culture medium with 10% CCK8 were added into 96-well plates for 2 h at 37 °C, the relative cell viability were measured at a certain wavenumber (450 nm) with Epoch Microplate Spectrophotometer (BioTek).

ROS Content Detection. MDA-MB-231 cells were seeded into 6-well plates and then pre-treated with or without 5 mM NAC for 2 h, and further incubated with Saline (negative control), AIO, AIO@NSe-Pt, iAIO@NSe-Pt, and iAIO@NSe-Pt+NAC for 6 hours. Subsequently, the ROS contents in cells were probed with DCFH-DA and subjected to Flow Cytometry assay using Reactive Oxygen Species Assay Kit (S0033, Beyotime) as manufacturer's instruction. Each group had repeated independently for three times and mean of FITC for three experiments were quantified as relative ROS content of cells. For confocal fluorescence assays, cells were cultured in 3.5 mm glass-bottom dish and treated with nanomaterials or saline as described for 6 h and then incubated with DCFH-DA probes, the ROS generation in cells were directly detected by confocal laser scanning microscopy.

Western Blot Experiment. MDA-MB-231cells were pre-treated with 64 μg/ml of Pt(IV) or iAIO@NSe-Pt for 18 h, and then were harvested with lysis buffer (50mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate and pre-mixed with protease inhibitor cocktail (B14001, Biomake)). After centrifugation at 12, 000g for 15 min at 4°C, the supernatant was collected and mixed with SDS-PAGE 5× loading buffer, boiling at 100°C for 5 min and subjected to SDS-PAGE. After electrophoresis, proteins were separated and blotted onto a PVDF membrane (Millipore). Membranes were probed with the specific primary antibody and then peroxidase-conjugated secondary antibodies. The bands were visualized by chemiluminescence.

Cellular ATP, NADPH and GSH Content Measurement. MDA-MB-231 cells were pre-treated with 32 μg/ml of Pt(IV), USIO, AIO@NSe-Pt, and iAIO@NSe-Pt for 12 h, then the cells were harvested and subjected to ATP, NADPH and GSH content measurement using ATP content measurement kit (BC0305, Solaibio), NADP+/ NADPH Assay Kit (S0179, Beyotime) and Micro Reduced Glutathione (GSH) Assay Kit (BC1175, Solaibio) as manufacturers' instruction.

H_2O_2 content and total SOD activity assessment.The Hydrogen Peroxide Assay Kit (S0038) and Total Superoxide Dismutase Assay Kit with WST-8 (S0101S) were obtained from Beyotime Biotechnology. 1×10^5 MDA-MB-231 cells were seeded in plates and then pre-treated with 32 μg/ml Pt(IV), AIO@NSe, AIO@NSe-Pt and iAIO@NSe-Pt for 12 h, the cells were harvested and subjected to H_2O_2 content and total SOD activity measurement according to manufacturers' instruction of Hydrogen Peroxide Assay Kit and Superoxide Dismutase Assay Kit. The relative content of SOD in cells were qualified by total protein density. In addition, the cellular H_2O_2 content also evaluated by staining with ROSGreen™ H_2O_2 probe Kit (MX5202, Maokang Biotech) according to the manufacturer's protocol. The images were collected with CLSM.

In Vivo Anti-tumorigenesis Mouse Model. All animal experiments were performed in compliance with the Ethics Committee Guidelines of Binzhou Medical University, and the animal protocols were approved by Laboratory Animal Center of Binzhou Medical University. Furthermore, any experimentation with animal subjects have achieved the informed consent of Laboratory Animal Center of Binzhou Medical University. Four to five weeks-old female

BALB/c mice were obtained from GemPharmatech Co Ltd (Nanjing, China). Four to five weeks-old female athymic nude mice (BALB/c mice) were obtained from GemPharmatech Co Ltd (Nanjing, China). Animals were randomly assigned to different groups as indicated, each group from MDA-MB-231 and 4T1 tumor models have 5 and 4 animals respectively. 1×10^6 of 4T1 cells in 100 μL of PBS (mixed with Matrigel (Corning) at 1:1 ratio) were injected subcutaneously into left leg of mice. Mice were treated with Pt(IV), AIO@NSe-Pt, iAIO@NSe-Pt, and iAIO@NSe-Pt+NAC at 3 days intervals via tail vein injection. Tumor size were measured once 3 days using a caliper one weekpost injection. Tumor volume were calculated using the standard formula $0.5\times L\times W^2$, where L is the longest diameter and W is the shortest diameter. Body weight of mice were also recorded. Afterwards, the mice were euthanized, tissues including hearts, livers, spleens, lungs, kidneys and tumors were removed, photographed and then subjected to tissue pathological analysis.

Evaluation of anti-angiogenesis effect of iAIO@NSe-Pt in vitro and in vivo. To evaluate the anti-angiogenesis effect of iAIO@NSe-Pt *in vitro*. HUEVC cells were cultured and used to perform the cell tubule formation assay. Briefly, Cultrex Basement Membrane Extract (BME, Cat. No.3432, Roche) was thawed overnight at 4℃ on ice, and 50 μL BME was added in each well of pre-cooled 96-well. The plates were placed at 37℃ for 30 min to form a thin gel layer with digestive endothelial cells (1×10^4) plated on top. The plate was incubated for 2-3 h at 37 ℃ in a 5% CO_2 incubator, and the formation of tube-like structures was observed and photographed under microscope. Whole mount assay staining with CD31 in situ were conducted to detect the phenotype of tumor-associated vessels. Fresh tumor tissues were fixed in 4% PFA overnight. The fixed tissues were cut into thin pieces and digested with protease K (10 mM) in a Tris buffer for 5 min, permeabilized with 100% methanol for 30 min, washed and blocked overnight with 3% milk in 0.3% Triton X-100 in PBS. Primary antibodies were incubated overnight at 4℃, followed by washing, blocking with 3% milk and incubation with fluorescent-conjugated secondary antibodies for 2 h at room temperature. Additional overnight washing was performed before mounting. The stained tissues were mounted with Vectashield mounting medium (H-1000, Vector Laboratories, USA). Fluorescent signals were examined

with a confocal microscope and quantitative analysis was performed with a Photoshop software.

Biodistribution of iAIO@NSe-Pt in vivo. Tumor-bearing mice were intravenously injected with Pt(IV), AIO@NSe-Pt, iAIO@NSe-Pt and iAIO@NSe-Pt+NAC at a concentration of 10 mg/kg normalized with Pt content. 12h post injection, mice were humanely sacrificed and the vital organs as well as tumor tissues were removed, weighed and homogenized. Then, the tissues were nitrated with the concentrated nitric acid solution for 48 h. Next, the resulting solution were diluted and filtering with 0.22 μm membrane. Finally, the Pt content were measured with ICP-MS.

Pathological Analysis of Tissues. The tissues were fixed in 15% buffered formalin, embedded in paraffin for histological analysis using IHC, IF and H&E staining. Ki67, cleaved-Caspase 3 and GPX4 antibodies were used in IHC and IF to evaluate the cell viability, apoptosis and ferroptosis potential of tumor tissues. And H&E staining of normal tissues were tested to assess the toxicity of nanomaterials in vivo. IF staining were performed using confocal laser scanning microscopy. The pathological analysis assay of tissues was performed as previously described [2]. The analysis was semiquantified by experienced pathologists in double-blind manner.

Biosafety evaluation in vivo by blood routine analysis. SD rats were divided into three groups and subjected to intravenous injection with saline, CDDP and iAIO@NSe-Pt at 2 mg/kg respectively. The blood of rats was collected at 24 h post injection, and the parameters of blood routine were analyzed by animal blood analyzer (Mascot, USA).

Lipid peroxidation and mitochondrial morphology detection in vivo. 1×10^5 4T1 cells in 100 mL of PBS were inoculated subcutaneously into the flanks of BALB/c mice. When tumors were palpable, they were intravenously injected with saline, Pt(IV), AIO@NSe-Pt, iAIO@NSe-Pt and iAIO@NSe-Pt+NAC at a dose of 2 mg/kg. One injection every day for a total of 5 times. Subsequently, tumors were dissected at 6 hours post the last injection, and frozen sections were made for subsequent staining. The frozen sections were stained with C11-BODIPY 581/591 working solution (10 μM diluted with ddH_2O) and incubated at 37 °C for 30 min, and the nucleus were staining with DAPI. Finally, the cellular fluorescence was monitored by CLSM. The fresh tumor tissues were also collected for preparation for transmission electron microscope. The tissues were fixed, embedded, sliced and stained with lead citrate. Finally, the

images of mitochondrial morphology in tumor cells were collected by electron microscope.

Reference.

[1] Cheng, J. J. ; Zhu, Y.; Xing, X.; Xiao, J. M.; Chen, H.; Zhang, H. W.; Wang, D.; Zhang, Y. Y.; Zhang, G. L.; Wu, Z. Y.; Liu, Y. Z. Manganese-deposited iron oxide promotes tumor-responsive ferroptosis that synergizes the apoptosis of cisplatin. *Theranostics,* **2021**, *11*, 5418-5429.

[2] Zhang, G. L.; Xie, W. T.; Xu, Z. W.; Si, Y. C.; Li, Q. D.; Qi, X. Y.; Gan, Y. H.; Wu, Z. Y.; Tian, G. CuO dot-decorated Cu@Gd_2O_3 core-shell hierarchical structure for Cu(I) self-supplying chemodynamic therapy in combination with MRI-guided photothermal synergistic therapy. *Mater. Horiz.*, **2021**, *8*, 1017-1028.

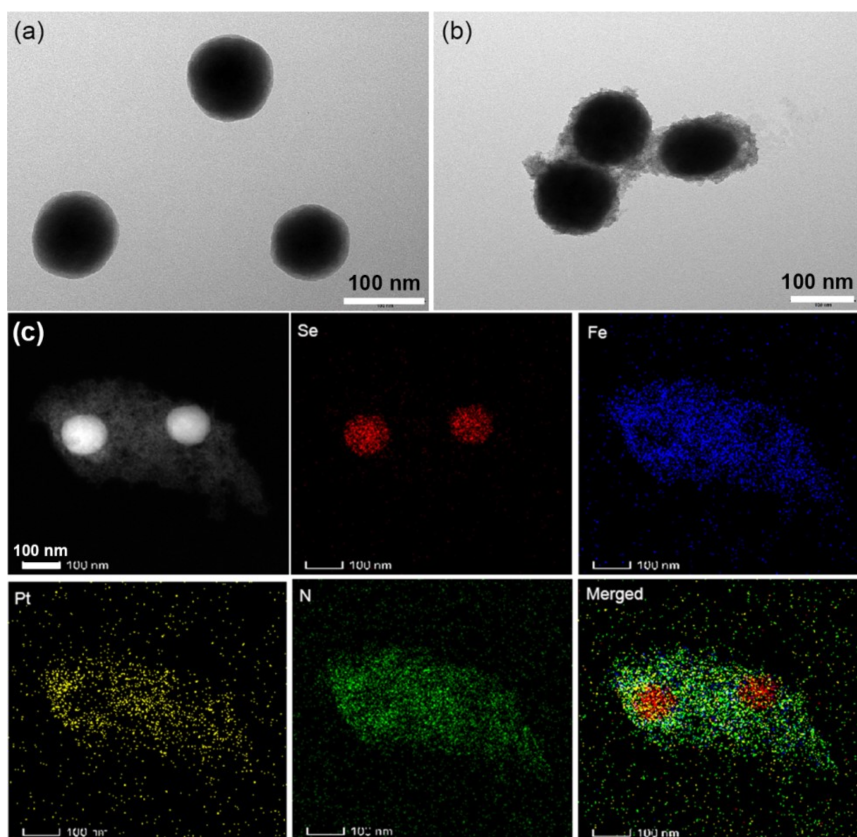

Figure S1. TEM images of (a) NSe and (b) AIO@NSe-Pt; (c) the elemental mapping of iAIO@NSe-Pt

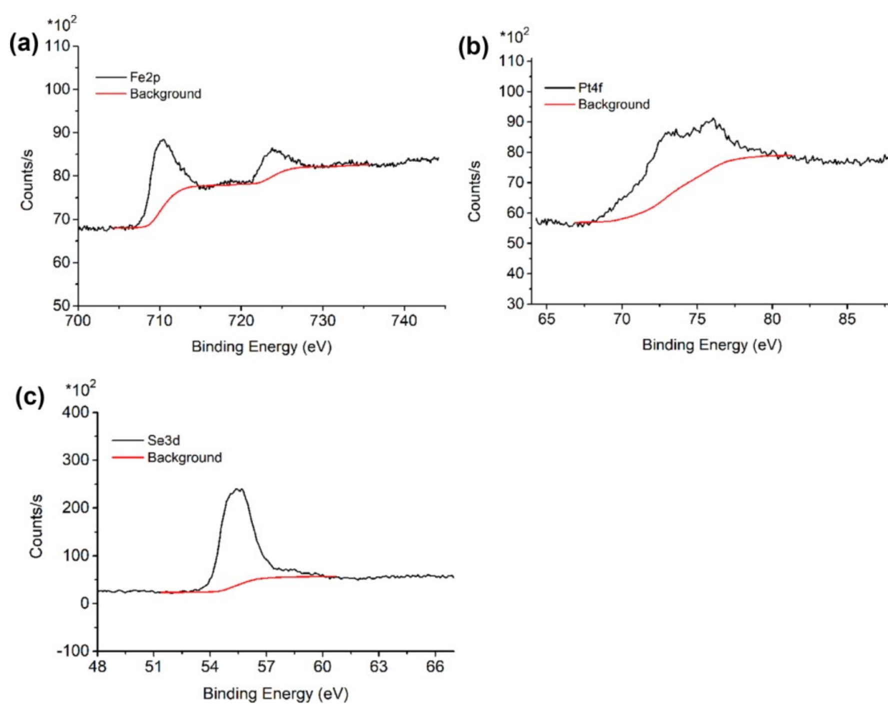

Figure S2. (a) XPS Fe2p spectra, (c) Pt4f spectra, and (d) Se3d spectra of iAIO@NSe-Pt.

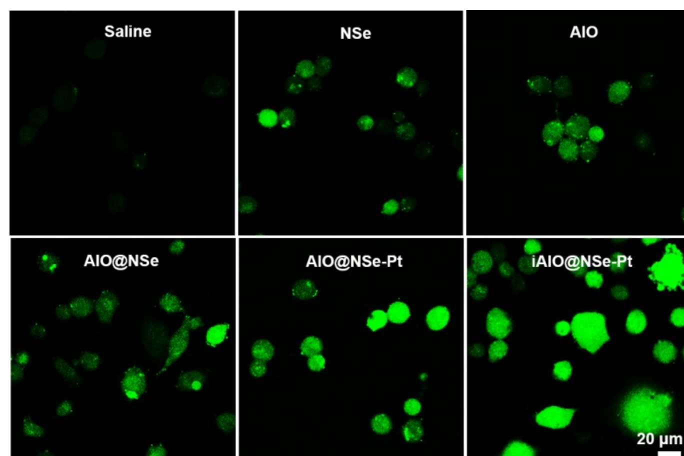

Figure S3. ROS generation in MDA-MB-231 cells treated with Saline, NSe, AIO, AIO@NSe, AIO@NSe-Pt, and iAIO@NSe-Pt. All CLSM images share the same scale bar.

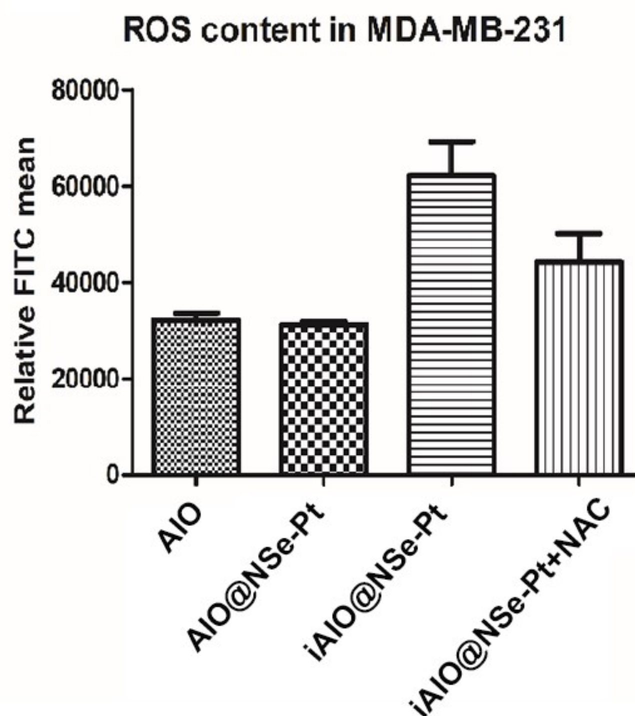

Figure S4. The relative content of ROS production in cells treated with different samples through flow cytometry analysis.

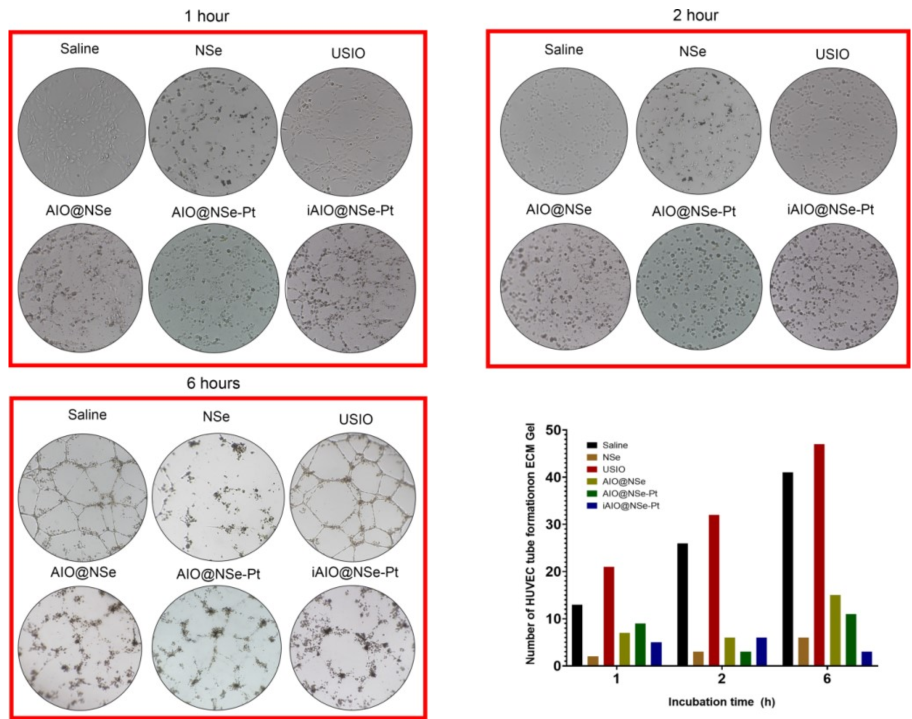

Figure S5. The tube formation of HUVEC cells treated with NSe, USIO, AIO@NSe, AIO@NSe-Pt, and iAIO@NSe-Pt.

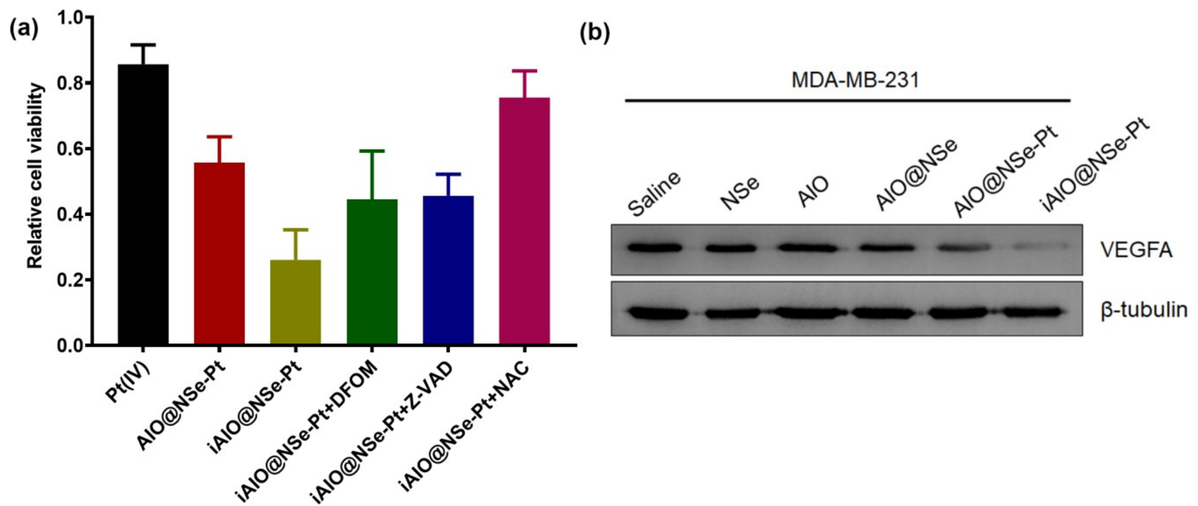

Figure S6. (a) Western blot analysis. The expression of VEGFA in MDA-MB-231 cells treated with different samples. (b) The viability of MDA-MB-231 cells treated with different samples for 24 hours.

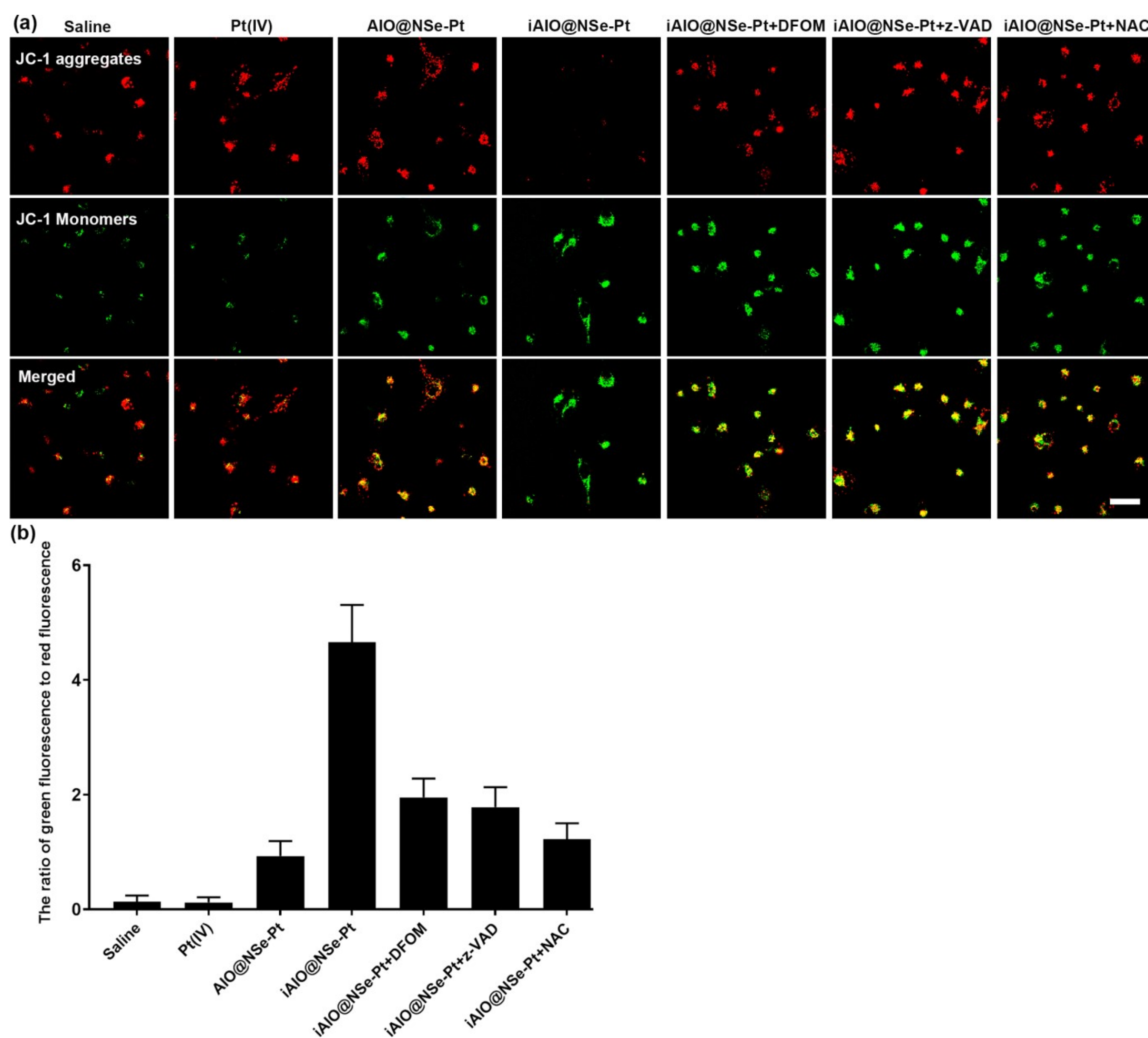

Figure S7. (a) The mitochondrial membrane potential observation of MDA-MB-231 cells treated with Pt(IV), AIO@NSe-Pt, iAIO@NSe-Pt, iAIO@NSe-Pt+DFOM, iAIO@NSe-Pt+z-VAD, and iAIO@NSe-Pt+NAC by CLSM. Scale bar represents 50 μm. (b) Corresponding ratio of green fluorescence to red fluorescence. All CLSM images share the same scale bar.

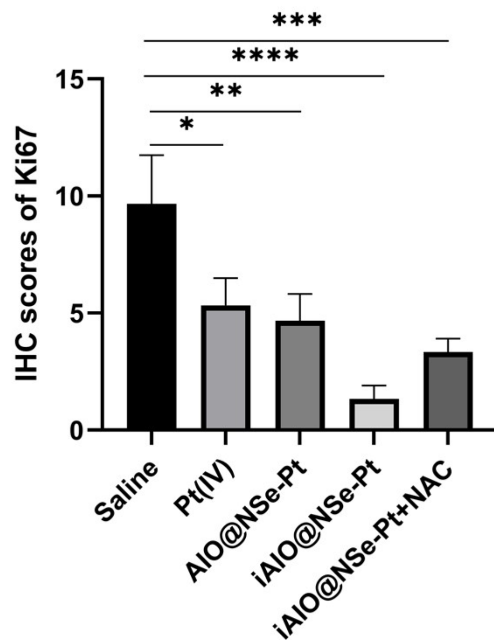

Figure S8. The IHC score of Ki67 in tumor slice after different samples treatment.

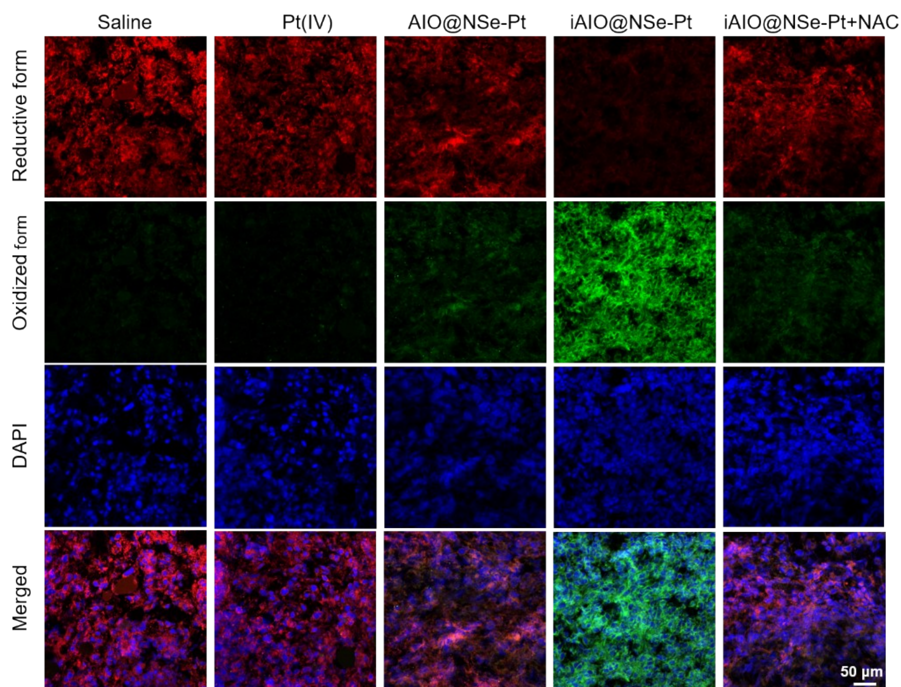

Figure S9. The lipid peroxidation production of tumor in mice treated with different samples.

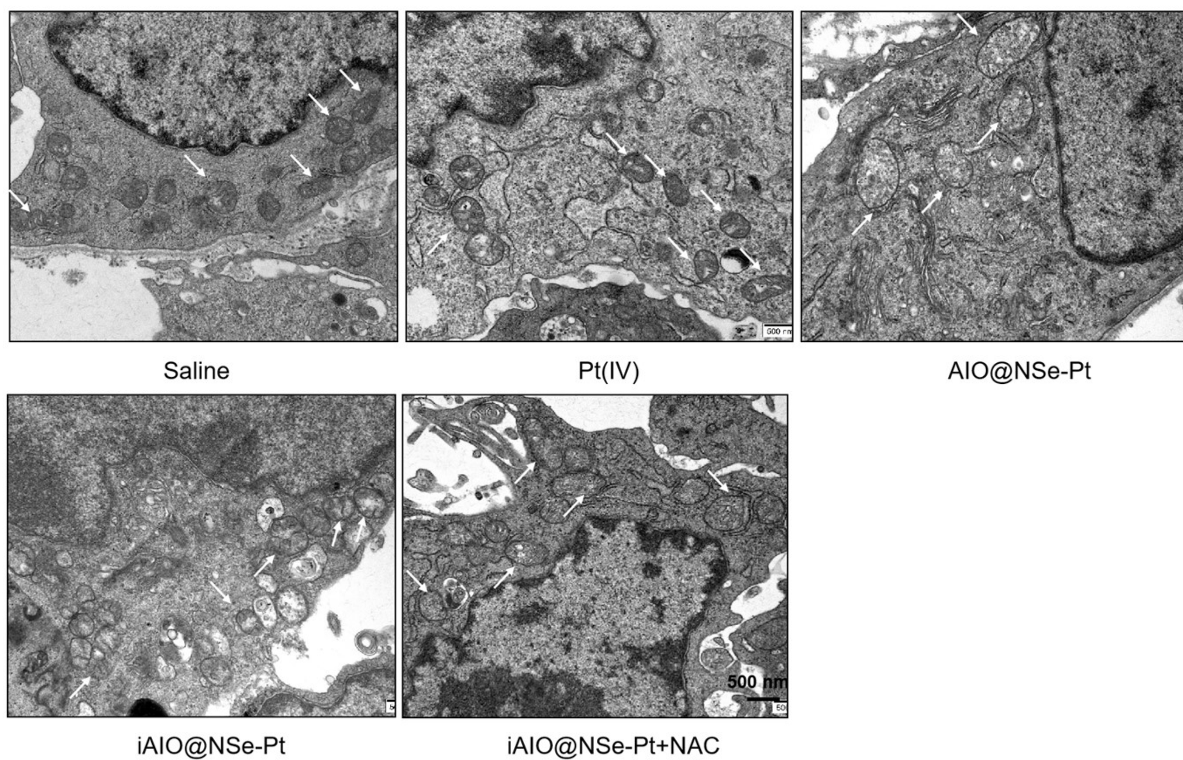

Figure S10. TEM images of tumor tissue after treatment with different samples.

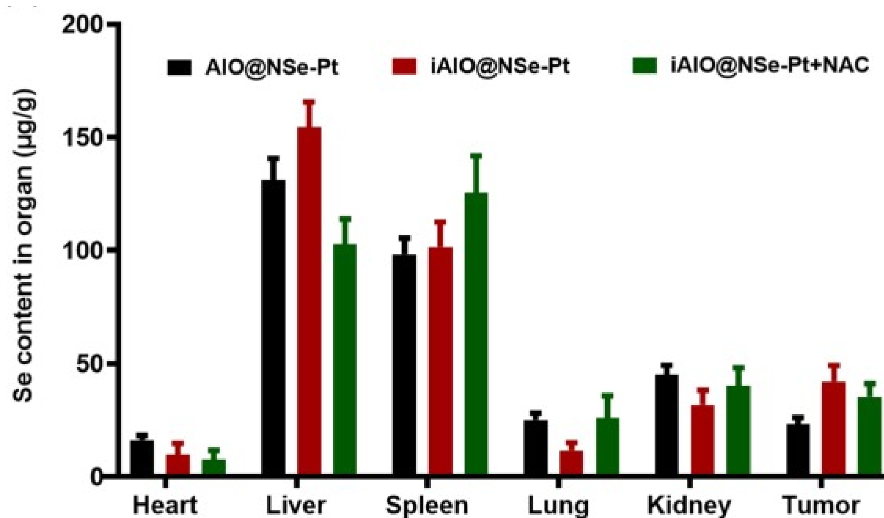

Figure S11. The biodistribution of Se element in mice at post-injection 24 h.

Table S1. The pharmacokinetic parameters of nanodrugs in mouse model.

	AUC 0-t (h*mg/L)	AUC 0-inf (h*mg/L)	Vd (L/kg)	CL (L/kg/h)	MRT (h)
PT(IV)	18.93	21.26	308.95	23.52	13.13
AIO@NSe-Pt	78.14	100.73	121.02	4.96	24.38
iAIO@NSe-Pt	156.43	167.92	43.58	2.98	14.64