Supporting Information

Two-photon excited peptide nanodrugs for precise photodynamic therapy

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Experimental Section

Materials

N, N-dimethylformamide (DMF) and Dichloromethane were purchased from Sinopharm Group Chemical Reagent Co. LTD. O-(benzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU), 4-methylmorpholine (NMM), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), Triisopropylsilane, Ethanedithiol trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Chemical Co. All Fmoc-amino acids and Wang-resins were obtained from GL Biochem. (Shanghai) Ltd. ABDA((9,10-anthracenediyl-bis (methylene)-dimalonic acid) was purchased from Sigma-Aldrich (Shanghai, China). PBS (phosphate buffered saline), FBS (fetal bovine serum), DMEM (dulbecco's Modified Eagle Medium) and trypsin were obtained from HyClone (Beijing, China). Calcein-AM, Alexa Fluor 488 and PI (propidium iodide) were also from Sigma-Aldrich (Shanghai, China). CCK-8(cell counting kit assay) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The HUVEC and MCF-7 cell lines were obtained from Cell Culture Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Science (Beijing, China). Ultrapure water was obtained from Milli-Q system with a resistivity of 18.2 MΩ cm^{-1}.

Preparation of BKC and BKC-NP

The BP-FFVLK-CREKA was prepared from C terminal to N terminal according to the standard solid-phase peptide synthesis techniques using Fmoc-coupling chemistry. Then BP motif was attached to the peptide by amidation reaction. Finally, the integral peptide BKC was cleaved from Wang resin beads and purified by reprecipitation for 3 times. Then BKC-NP was obtained by mixed different concentrations of BKC-NP DMF solution with 99% water, the obtained BKC NPs were stored at 4 ℃.

Apparatus and Characterization

The BKC-NP was characterized by HT7700 transmission electron microscopy (TEM). Particle size and distribution of BKC-NP were recorded by a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern, UK). The UV-vis spectra were measured on a SHIMADZU-2600 spectrophotometer. The fluorescence spectrum of BKC-NP was conducted with a Hitachi F-4500 fluorescence spectrometer. The confocal laser scanning microscopy (CLSM) images of samples were collected with an Olympus FV1000 instrument, which was equipped with one- (405, 488, 559 and 633 nm) and two-photon lasers (Spectra-Physics, MaiTai Deepsee®). The fluorescence imaging in vivo was obtained using a small-animal imaging system (Spectrum CT, PerkinElmer).

Detection of ROS

We employed the chemical reagent ABDA to assess the capability of singlet oxygen generated by drug. 25 μL ABDA/DMSO (10 mM) was homogeneously mixed with 1.5 mL BKC-NP (30 μM), then irradiated using a xenon lamp with a 405 nm cutoff filter for 30 min. The two-photon laser of 800 nm induced ROS of BKC-NP (0.36 mM) was also detected, the irradiation time was lasted for 30 min, and the laser power was 50 mW, at different intervals, the solution was detected by UV/Vis, spectra of mixtures were collected from 300 to 450 nm at different intervals. The

control experiments were carried out under the same conditions without samples.

Endocytosis study of BKC-NP

HUVEC and MCF-7 cells were seeded into 20 mm Petri confocal dishes for overnight. 200 μL BKC-NP solution (30 μM) was added in and incubated for another 5 h. After that, the cells were washed twice with PBS to remove the untaken drugs. The images were obtained with confocal laser scanning microscope with a 405 nm laser excitation (60 × objective lens).

Detection of cytotoxicity

The cells were cultured in 96-wells culture plates with the density of 1×10^5 cells/well in 5% CO_2 at 37 °C overnight. Then the culture medium was removed and different concentrations of BKC-NP (0, 15, 30, 60, 90, 120 μM) were added for further incubation for 5 h. Subsequently, the 96-wells culture plates were irradiated by two-photon laser of 800 nm (1 W) or a xenon lamp with a 405 nm cut-off filter for 20 minutes, and continued culturing for another 20 h. The cells were washed twice with PBS to remove the untaken drugs. The viability of HUVEC cells were evaluated by CCK-8 assay. And the control group was tested without light.

Also, HUVEC cells were cultured in a 20 mm confocal dish and incubated at 37 °C for overnight. Then, the cells were treated with BKC-NP (120 μM) at 37 °C for 5 h. After that, the cells were washed twice with PBS, 10 μL Calcein-AM and 10 μL PI were added into the confocal dish. Then the cells were irradiated with 800 nm two-photon laser for 30 min and incubated for another 12 h. The confocal images were obtained with 559 nm laser at 8% intensity. And the control group was without drug.

Tumor models and live subject statement

The protocol of animal experiments was conducted in accordance with the guidelines approved by the Institutional Ethical Committee of Animal Experimentation of National Center for Nanoscience and Technology. All researchers were informed and agreed with the aims of this experiment. BALB/c mice (female, ~15 g) were provided by the Beijing Charles River Laboratory Animal Technology Co., Ltd. 100 μL MCF-7 cells (1×10^7 cells mL^{-1}) were inoculated subcutaneously into the right hind leg of each mice. The mice were proceeded to further experiments when the tumor has reached a certain volume.

In vivo blood vessel imaging and closure experiment

When the tumor reached a volume about 200 mm^3 (tumor volume = 1/2 (length × $width^2$)), the mice were firstly anaesthetized by intraperitoneal injection of 5% chloral hydrate (0.08 mL kg^{-1}). An inverted confocal microscope was used for bioimaging of the peripheral blood vessel of tumor and the ear blood vessel via two-photon excitation. Then the mice were injected with 200 μL BKC-NP (500 μM) via tail vein. Photos were obtained at predetermined time points (20, 40 and 60 min).

For in vivo vascular occlusion experiments, the mice were firstly anaesthetized with 5% chloral hydrate (0.08 mL kg^{-1}). Then, the mouse was injected with 200 μL BKC-NP (500 μM) via tail vein and the control group without BKC-NP. An oil-immersion objective lens (60 ×) was used to irradiate the peripheral blood vessel of tumor. A section of the blood vessel was irradiated as a series images, each 1 μm apart through the depth, and total scan depth is 24 μm. After 2 minutes continues scan

with an 800 nm two-photon laser at 7% intensity (0.5 mW), the two-photon images of before and after were obtained.

Biodistribution and antitumor efficiency in vivo

When the tumor grows until a volume about 200 mm^3, the mice were intravenously injected with 200 μL BKC-NP (500 μM). The mice were sacrificed at different intervals (20, 40, 90, 180 min) after the injection. Then the organs (heart, lung, kidney, liver, spleen and tumor) were collected for imaging by a small animal living functional structure imaging system. The excitation wavelength was 430 nm and the emission wavelength was 520 nm.

When the tumor diameter reached 4~6 mm, the mice were separated into four groups (n = 4) randomly. The first to fourth groups were control, BKC-NP (200 μL, 500 μM), laser and laser + BKC-NP (200 μL, 500 μM), respectively. 90 min after the injection on day 1 and 4, the third and fourth groups were anesthetized and irradiated by 800 nm fs Ti: sapphire laser (Spfire Ace-1001HP, Spectra-Physics) for 7 min (3.5 W cm^{-2}). On day 8 was 8 min with 4 W cm^{-2} laser. The laser spot was 6 mm. After the treatment, the tumor volumes and body weights were recorded every 2 days.

Statistical analysis

Data in this experiment was presented as mean ± standard deviation (SD), and statistical analyses were performed with LSD Duncan, one-way ANOVA analysis, $^{**}P < 0.01$.

Supplementary data:

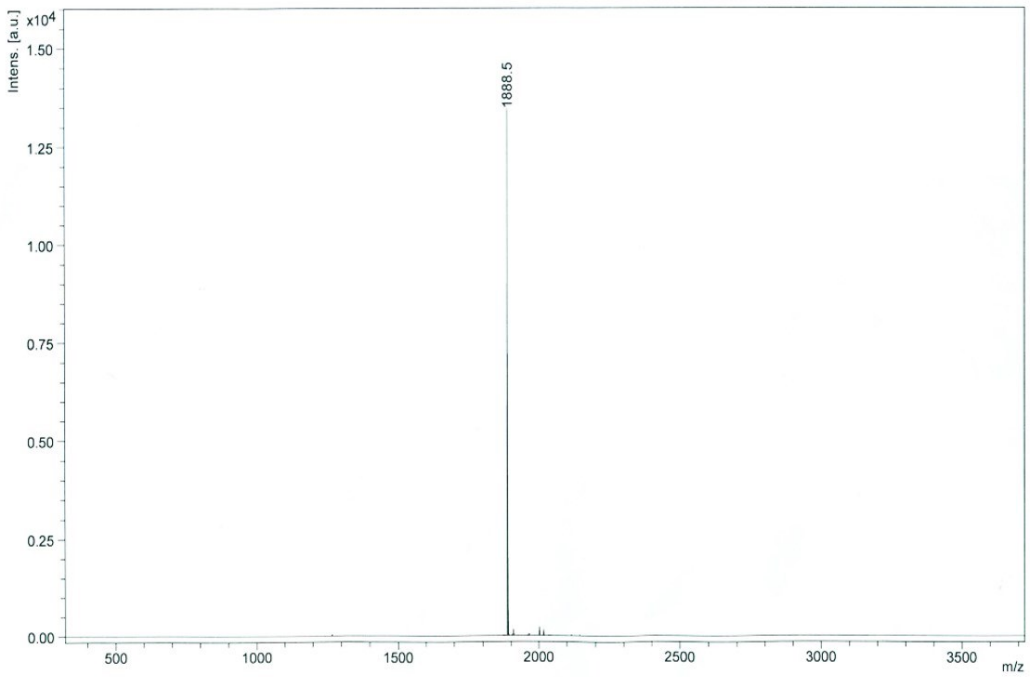

Figure S1. The molecular structure of BKC confirmed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra.

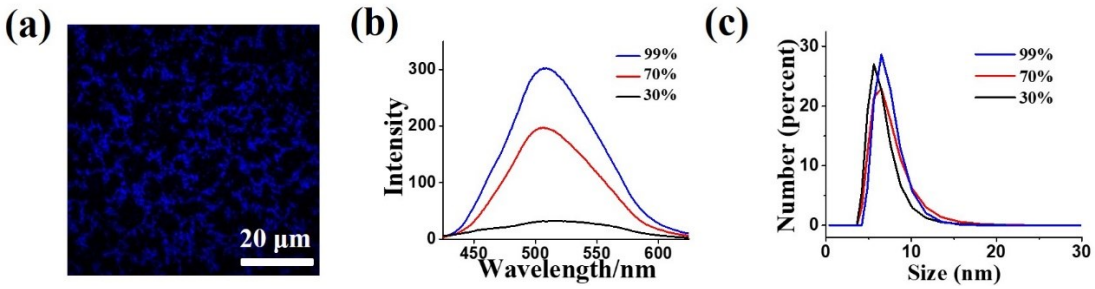

Figure S2. (a) CLSM image of BKC-NP excited with one-photon laser (405 nm). (b) Fluorescence intensity of BKC-NP solution with different water fractions, the wavelength was collected from 425 to 625 nm. (c) DLS results of BKC-NP solutions with different water fractions.

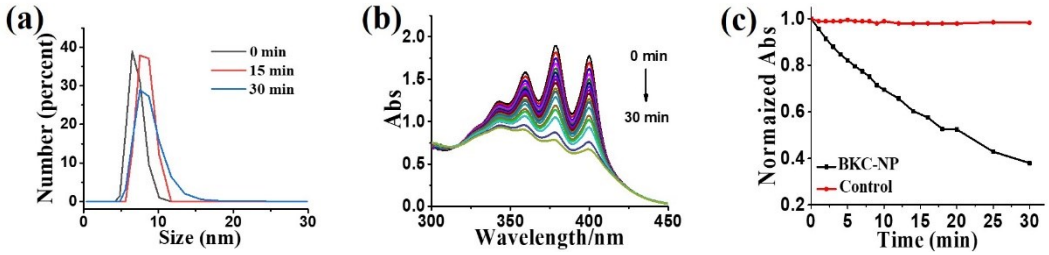

Figure S3. (a) DLS results of BKC-NP solutions treated with different irradiation times of two-photon laser of 800 nm. (b) UV absorbance values of ABDA solution incubated with BKC-NP treated with 405 nm laser for different times. (c) Normalized absorption values of BKC-NP and water under the same irradiation condition.

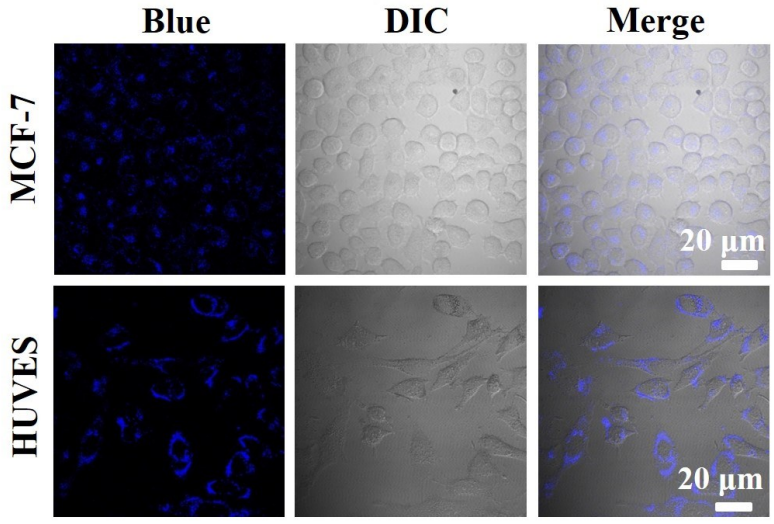

Figure S4. Cell endocytosis of BKC-NPs. Endocytosis effect of MCF-7 and HUVES cells incubated with BKC-NPs for the same time.

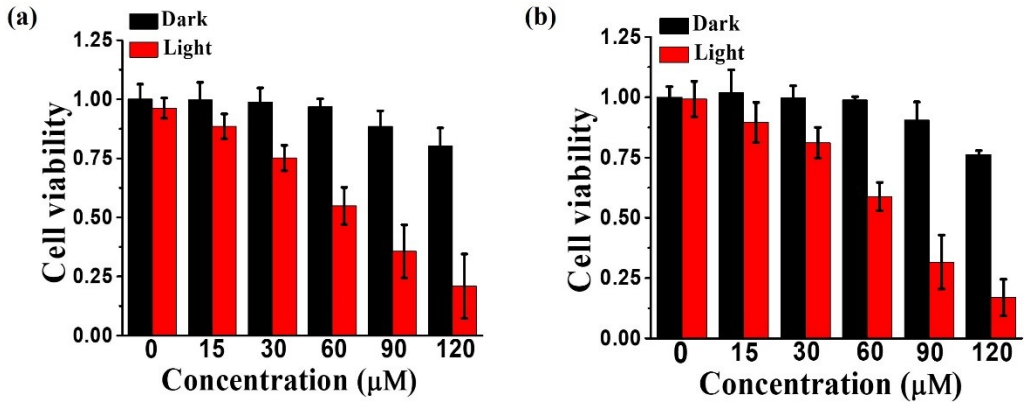

Figure S5. Cell viability of HUVEC cells treated with BKC-NP solutions of different concentrations. (a) The light group was performed under irradiation of 800 nm laser and the control group was incubated in the dark. (b) The light group was treated with 405 nm laser and the control group was treated with no laser.

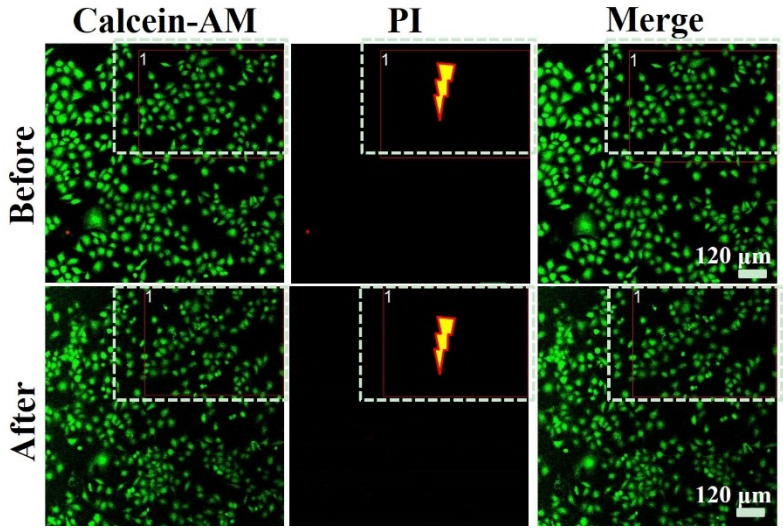

Figure S6. CLSM images of HUVEC cells irradiated with two-photon laser (800 nm), the cells were incubated without BKC-NPs, photographs were obtained before and after the irradiation.

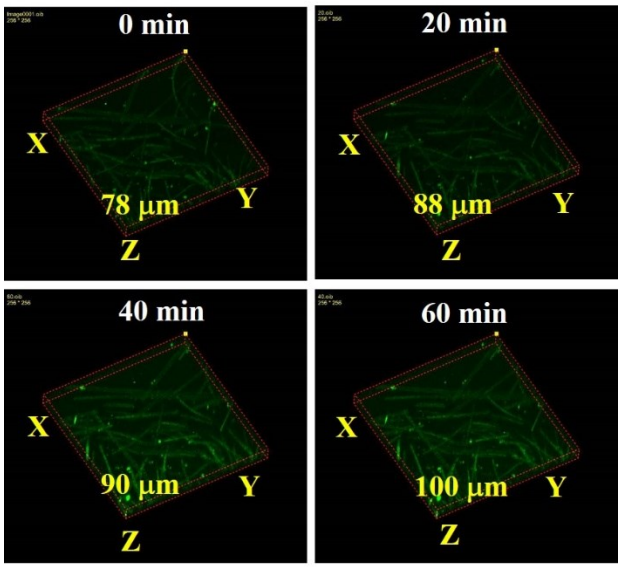

Figure S7. 3D imaging depth pictures of BKC-NP in tumor tissue collected by CLSM. After the tail vein injection, images were acquired at 0, 20, 40, 60 mins.

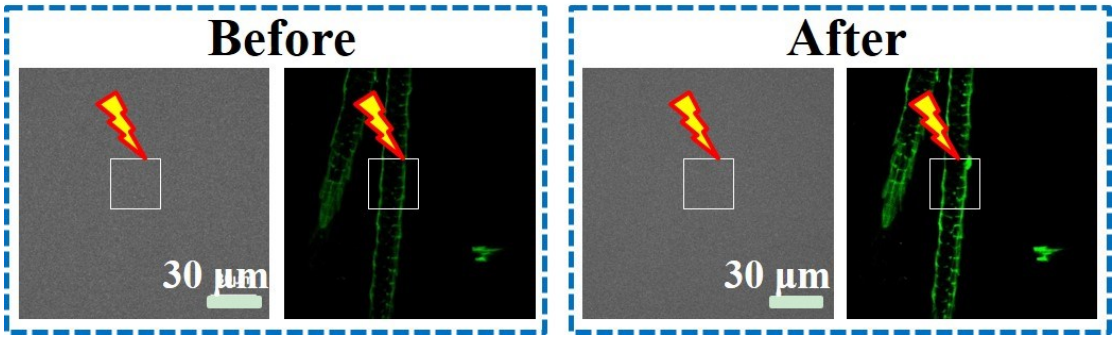

Figure S8. Selected region of ear blood vessels irradiated by two-photon laser. Images were obtained before and after the treatment, and the mouse was treated with no nanodrugs.

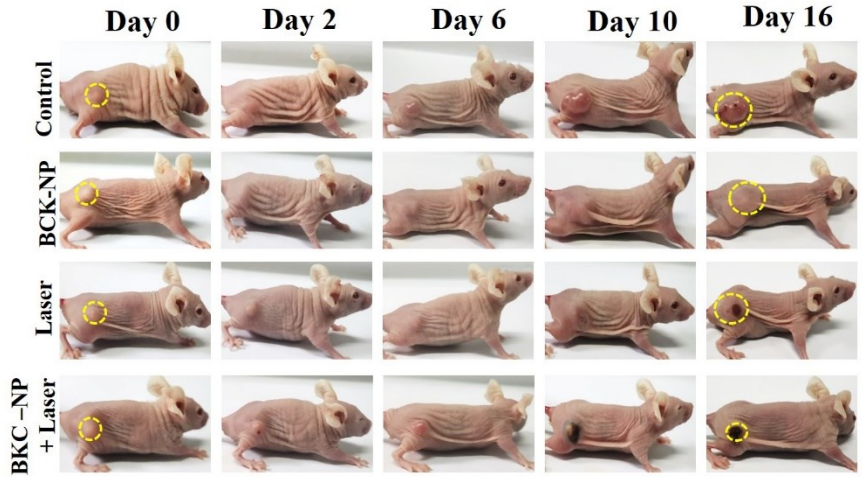

Figure S9. Anti-tumor investigation in vivo. Mice were randomly separated into four groups and treated with BKC-NP, laser, BKC-NP + laser or no treatment during the observation period, the observation time was 16 days. Tumors were circled by yellow lines.