Supplementary Information

Semiconducting polymer-based nanoparticles for photothermal therapy at second near-infrared window

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Experiments

Materials

Pluronic *F-127* (F-127) was purchased from Sigma Aldrich Co., Ltd.. PCL-PEG-Folate was purchased from Xi'an Ruixi Biological Technology Co., Ltd. 4', 6-diamidino-2-phenylindole (DAPI) and Cell Counting Kit-8 (CCK-8) were obtained from Dojindo Molecular Technologies. LIVE/DEAD® Viability/Cytotoxicity Kit and Annexin V-Fluoroisothio cyanate (FITC)/propidium iodide (PI) apoptosis detection kit were provided by Invitrogen. All other chemicals, if not specified, were used as received without further purfification. All the reactions were carried out under nitrogen atmosphere.

Synthesis of P(AcIID-DPP)

A solution of 2,5-bis(2-octyldodecyl)-3,6-bis(5-(trimethylstannyl)thiophen-2-yl)-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione (0.1187 mg, 0.1 mmol), (E)-6,6'-dibromo-1,1'-bis(2-ethylhexanoyl)-[3,3'-biindolinylidene]-2,2'-dione (0.0673mg, 0.1 mmol),

$Pd_2(dba)_3$ (1.8 mg, 0.0020 mmol), and P(o-tolyl)$_3$ (2.4 mg, 0.0080 mmol) in anhydrous toluene (8 mL) were placed in a Schlenk tube. The resulting mixture was subjected to three cycles of evacuation and admission of nitrogen and then stirred at 110 °C for 48 h.[1] After polymerization, the reaction mixture was cooled down to room temperature and poured into stirring methanol to precipitate the polymer product. The precipitated polymer Poly(2,5-bis(2-octyldodecyl)-3,6-di(thiophen-2-yl)-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione-alt-(E)-1,1'-bis(2-ethylhexanoyl)-[3,3'-biindolinylidene]-2,2'-dione) (P(AcIID-DPP)) was collected by filtration and purified by sequential Soxhlet extractions with methanol and hexane each for 24 h. The remaining residue was extracted with chloroform, concentrated and poured into stirring methanol to precipitate the final polymer product as a black solid (126 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.95-8.60 (br, 4H), 8.30-8.00 (br, 2H), 7.70-7.30 (br, 4H), 4.20-3.10 (br, 6H), 2.00-0.65 (br, 102H). GPC (THF): Mn = 61.2 kPa, PDI = 1.93.

Preparation of DPP-IID-FA NPs and DPP-IID NPs

DPP-IID-FA NPs were prepared through emulsion method. Briefly, P(AcIID-DPP) (1 mg), PCL-PEG-FA (0.5 mg) and F-127 (20 mg) were dissolved into 1 mL of CH_2Cl_2. The obtained solution was injected into 10 mL PVA solution (1 mg·mL^{-1}) and emulsified by sonication for 2 min (work 9.5 s and rest 0.5 s) at 325 W output using a microtip probe sonicator (JY92-IIN, Scientz biotechnology, Ningbo, China).[2, 3] The mixture was further stirred under room temperature for 2 h to evaporate the CH_2Cl_2, and then centrifuged at a speed of 24, 000 rpm for 20 min to obtain the DPP-IID-FA nanoparticles.

The DPP-IID NPs were prepared by the same method, but with PCL-PEG to instead of PCL-PEG-FA. To evaluate the cellular uptake ability of DPP-IID and DPP-IID-FA, the coumarin 6 was doped into nanoparticles to obtain DPP-IID/CU and DPP-IID/CU-FA NPs.

Characterization

Nuclear magnetic resonance (NMR) spectra were conducted on a Bruker Ultrashield 400 Plus NMR spectrometer. Gel permeation chromatography (GPC) was

carried out on a Waters e2695 liquid chromatography instrument using tetrahydrofuran as eluent with polystyrenes as standards. UV-vis-NIR spectra were recorded on a Perkin Elmer Lambda 950 UV-Vis-NIR spectrophotometer. Dynamic light scattering (DLS) was conducted on Zetasizer Nano ZS (Malvern Instruments Ltd., UK). TEM image was provided by transmission electron microscopy (TEM, FEI Company, Hillsboro, OR) with an accelerating voltage of at 200 kV. Laser-confocal scanning imaging of Hela cells was executed by using confocal microscope Zeiss LSM780.

Photothermal effect and photostability of DPP-IID-FA NPs under 1064 nm laser irradiation

The aqueous solution of DPP-IID-FA with different concentrations (0, 2.5, 5.0, 10, 20, and 40 μg·mL^{-1}) in centrifuge tube was irradiated by a 1064 nm laser (1 W/cm^2) for 5 min. The changes in temperature were recorded by a thermocouple microprobe (ϕ = 0.5 mm) (STPC-510P, Xiamen Baidewo Technology Co., China). The photostability of DPP-IID-FA was evaluated by monitoring its photothermal effect after five cycles of laser on/off with 1064 nm laser irradiation. Briefly, the DPP-IID-FA solution (20 μg·mL^{-1}) was irradiated by a 1064 nm diode laser at 1 W/cm^2 for 5 min (laser on), followed by naturally cooling to room temperature for 15 min (laser off). The laser on and laser off cycles were repeated for five times, and the change in temperature was monitored using a thermocouple microprobe as described above.

Calculation of the photothermal conversion efficiency

To evaluate the photothermal conversion efficiency, the temperature change of the aqueous dispersion (40 μg· mL^{-1}) was recorded as a function of time under continuous irradiation of the 1064 nm laser with a power density of 1.4 W·cm^{-2} until the solution reached a steady-state temperature.

The photothermal conversion efficiency, η, was calculated using Equation 1 described by previous reports,[4, 5] where h is the heat transfer coefficient, A is the surface area of the container, Tmax is the equilibrium temperature, T_{Surr} is ambient temperature of the surroundings, $\Delta T_{max} = T_{max} - T_{Surr}$, I is incident laser power (2 W cm^{-2}), and A_λ is the absorbance of DPP-IID-FA nanoparticles at 1064 nm. Qs is the heat associated with the light absorbance of the solvent, which is measured independently to be 25.2 mW using deionized water without nanoparticles.

$$\eta = \frac{hA\Delta T_{max} - Q_s}{I(1-10^{-A_\lambda})} \qquad (1)$$

The value of hA is derived according to Equation 2:

$$\tau_s = \frac{m_D C_D}{hA} \qquad (2)$$

Where τ_s is the time constant of sample system, m_D and C_D are the mass (1 g) and heat capacity (4.2 J g^{-1}) of deionized water used as the solvent, respectively.

In order to obtain the hA, herein introduce θ, which is defined as the ratio of ΔT to ΔT_{max}:

$$\theta = \frac{\Delta T}{\Delta T_{max}} \qquad (3)$$

hA can be determined by applying the linear time data from the cooling period versus –Lnθ (Fig. 2e-f). Substituting hA value into Equation 1, the photothermal conversion efficiency (η) of T DPP-IID-FA nanoparticles can be calculated:

$hA = m_D C_D/\tau_s = 4.2$ J/260; $A\lambda = 0.469$; $\tau_s = 207.8$; $I = 1.4$ W cm^{-2}; $\Delta T_{max} = 33.9$ °C

$\eta = ((4.2/207.8)\times 33.9 - 0.0252)/(2\times(1-10^{-0.0.469})) = 49.5\%$

Cell Culture

HeLa cervical adenocarcinoma epithelial cells (Hela) were purchased from the American Type Culture Collection (ATCC). Hela cells were cultured in DMEM

(Dulbecco's modified Eagle medium) (GIBCO) with 10% FBS (fetal bovine serum) (GIBCO) and 1% antibiotics (penicillin–streptomycin) (Corning) in a humidified environment which contains 5% CO_2 and 95% air at 37 °C.

Cellular internalization analysis

To investigate the intracellular uptake of DPP-IID-FA nanoparticles, the Hela cells were plated into the confocal laser scanning microscopy (CLSM)-specific dishes (35 mm × 10 mm, Corning Inc., New York) and allowed to adhere for 24 h. Then, the cells were divided into following four groups: (1) control, (2) DPP-IID/CU-FA, (3) DPP-IID and (4) DPP-IID/CU-FA+Hela cells pre-treated by FA (1 mg/mL) for 2 h. Afterwards, the treated cells were washed with PBS for 3 times, and then fixed with 4% formaldehyde for 10 min. Subsequently, the cell nuclei were stained with DAPI (5 μM) for another 10 min. Finally, the cells were imaged by CLSM (Zeiss LSM780) with 488 nm laser excitation for FITC and 405 nm laser excitation for DAPI.

In Vitro PTT Therapy

DPP-IID-FA nanoparticles in PBS were diluted to various concentrations by DMEM. Then, the Hela cells were cultured in 96-well plates and divided into four groups: (1) DPP-IID-FA, (2) DPP-IID-FA+Laser; (3) DPP-IID+Laser and (4) DPP-IID-FA+Hela pre-treated by FA+Laser. The group (1) was maintained in darkness, while other groups were irradiated by laser (1064 nm, 0.8 $W \cdot cm^{-2}$, 5 min). Afterwards, the cells were incubated at 37 ºC with 5% CO_2 and 95% air for 24 h. Finally, the cell culture medium was replaced by 100 μL fresh medium, and then 10 μL of CCK-8 solution was added in each well. After further incubation the cells at 37 °C for 2 h, the absorbance at 450 nm in each well was recorded by the SpectraMax M5 Microplate Reader (Molecular Devices, USA). The cell viability was calculated by the following formula:

$$\text{Cell viability} = (OD_1 - OD_{blank}) / (OD_0 - OD_{blank}) \times 100\%.$$

Here, the OD_1 and OD_0 represent the absorbance values of non-treated cells and treated cells, respectively, while the OD_{blank} is the absorbance of free CCK-8 itself. The cell viability was presented as the average value from four independent experiments.

Live/Dead Cell Staining Assay

Hela cells were seeded in 96-well plates at a density of 2.5×10^4 cells per well and divided into six groups: (1) Control, (2) DPP-IID-FA, (3) Laser, (4) DPP-IID-FA+Laser, (5) DPP-IID+Laser and (6) DPP-IID-FA +Hela pre-treated by FA+Laser. After laser irradiation, the cells were incubated at 37 ºC with 5% CO_2 and 95% air for 24 hours. Finally, all cells were stained by LIVE/DEAD® Viability/Cytotoxicity Kit in DMEM medium and incubated for 15 min at 37 °C with 5% CO_2. Finally, the cells were observed by a fluorescence microscope (Zeiss Axio Vert.A1, Germany). Excitation of the calcein-AM and PI was performed by lasers at 488 and 543 nm, respectively.

Apoptosis Evaluation by Flow Cytometry

Hela cells were seeded in 6-well plates at a density of 3×10^5 cells per well. After 24 h incubation, the cells were divided into six groups: (1) Control, (2) DPP-IID-FA, (3) Laser, (4) DPP-IID-FA+Laser, (5) DPP-IID+Laser and (6) DPP-IID-FA +Hela pre-treated by FA+Laser. After laser irradiation, the cells were incubated at 37 ºC with 5% CO_2 and 95% air for 24 hours. Afterwards, all of the cells were washed with PBS for 3 times, digested by trypsin, and collected by centrifugation. After being further washed with PBS for 3 times, the cells were re-suspended in 0.5 mL of annexin binding buffer. After that, all cells were stained in PI and Annexin-V-FITC containing binding buffer for 15 min and finally detected by flow cytometry (BD FACSAria TM III).

In vivo Biosafety Evaluation

For *in vivo* biosafety evaluation, the BALB/c mice were intravenously injected with DPP-IID-FA nanoparticles at the dosage of 2.0 mg/mL for 150 μL. After 7 days of injection, the mice were sacrificed and the blood and major organs including heart, liver, kidney, lung and spleen were collected. The biochemical indicators of liver functions (AST, ALP, TP, ALB and ALT) and kidney functions (UA and Cr) were evaluated; meanwhile, the pathological evaluation of major organs (H&E staining) were also conducted.

In Vivo PTT Therapy

The *in vivo* experiment was performed following our previous reported protocol. The animal studies have been conducted strictly under the guidelines of "National animal management regulations of China", and approved by the Animal Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University. The tumors were obtained by subcutaneously injecting of 5×10^6 Hela cells (suspended in 100μL PBS) into the right armpit region of the female nude mice. After the tumor size achieved approximately 50 mm^3, the Hela-tumor-bearing mice were randomly divided into 5 groups with five mice in each group as follows: (1) control (PBS), (2) DPP-IID-FA, (3) 1064 nm laser, (4) DPP-IID + 1064 nm laser and (5) DPP-IID-FA + 1064 nm laser. Mice bearing Hela tumors were intravenously injected with 150 μL (2.0 mg/mL) of nanoparticles, and mice treated with the same volume of saline were used as the control. The temperature changes at the tumor sites in group 3-5 were recorded by an infrared thermal camera during the irradiation, and the infrared thermal images were taken at the same time. The body weight and the tumor volume of the mice were measured every 2 days by using an electronic balance and a vernier caliper, respectively. Moreover, the hematoxylin and eosin (H&E) as well as the immunofluorescence staining were carried out to evaluate the tissue destruction and cell apoptosis after therapy.

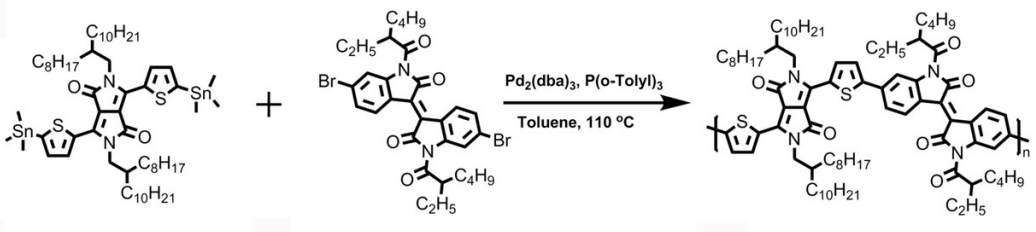

Fig. S1 Synthetic routes of P(AcIID-DPP). Reagents and conditions: $Pd_2(dba)_3$, P(o-tolyl)$_3$, anhydrous toluene, 110 ºC, 48 h.

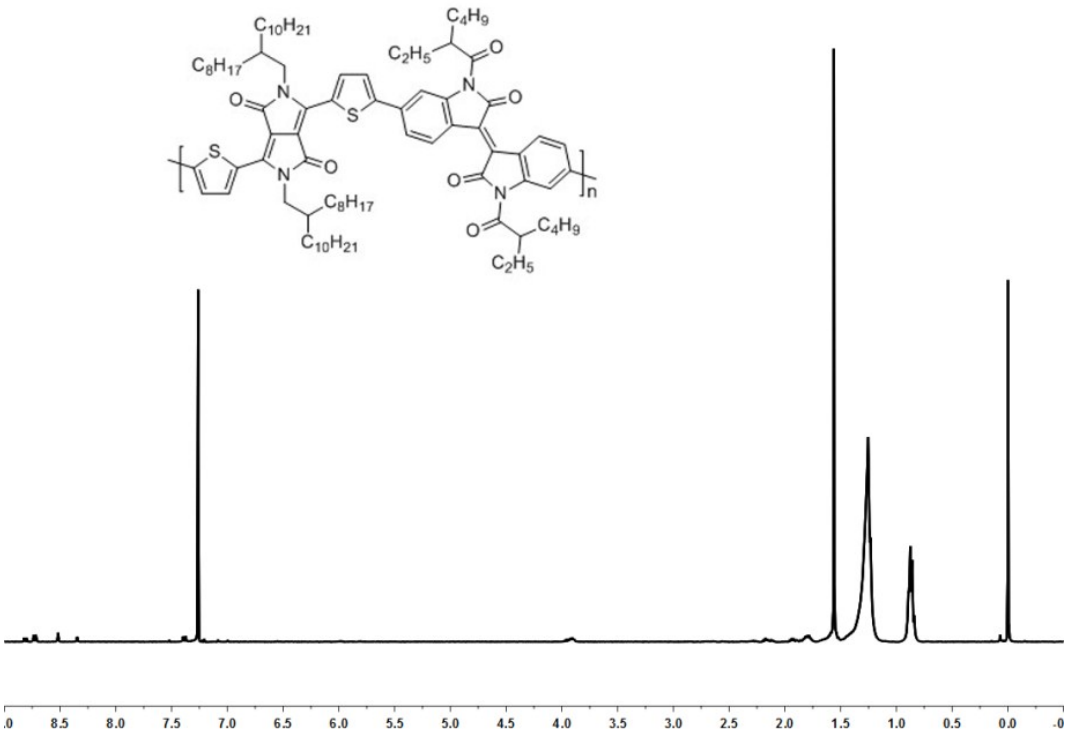

Fig. S2 ^{1}H NMR spectrum of P(AcIID-DPP).

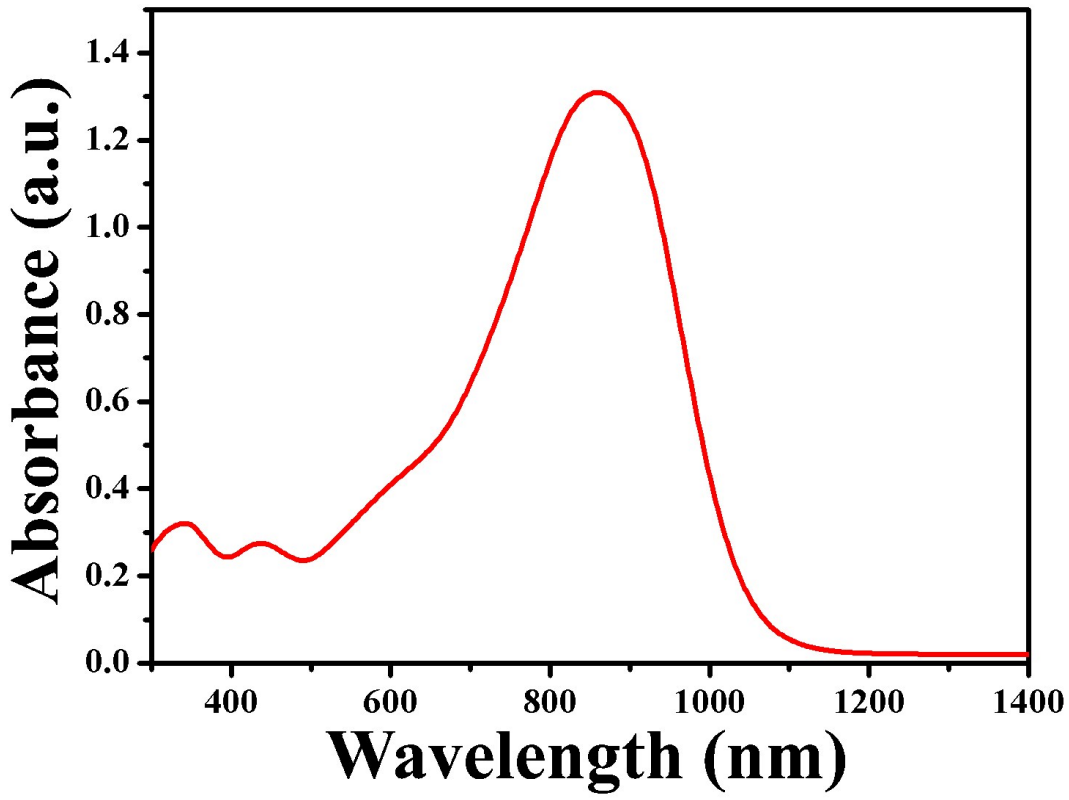

Fig. S3 The UV-vis-NIR spectrum of P(AcIID-DPP) in chloroform solution.

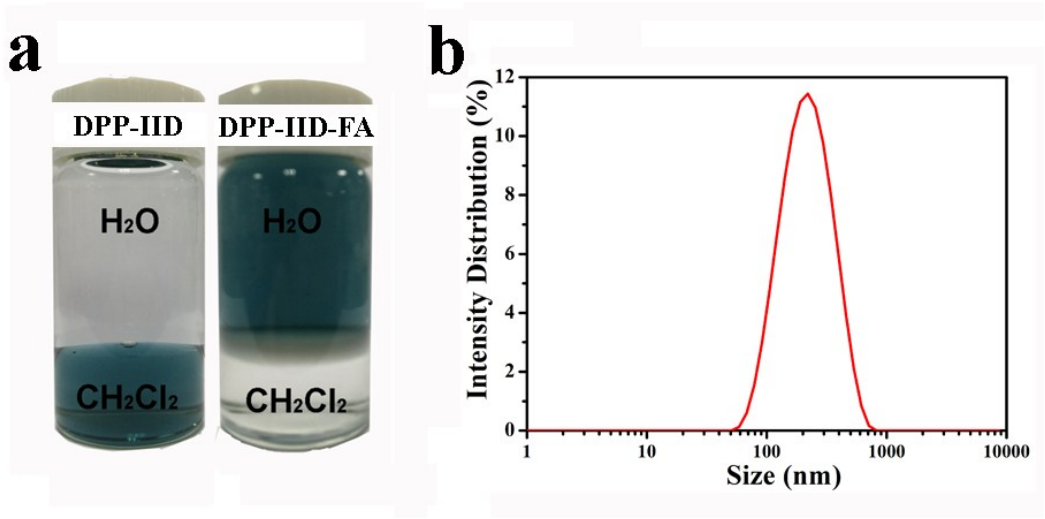

Fig. S4 (a) Photograph of the P(AcIID-DPP) and DPP-IID-FA nanoparticles in H_2O/CH_2Cl_2. (b) Dynamic light scattering (DLS) measurement of the DPP-IID-FA nanoparticles.

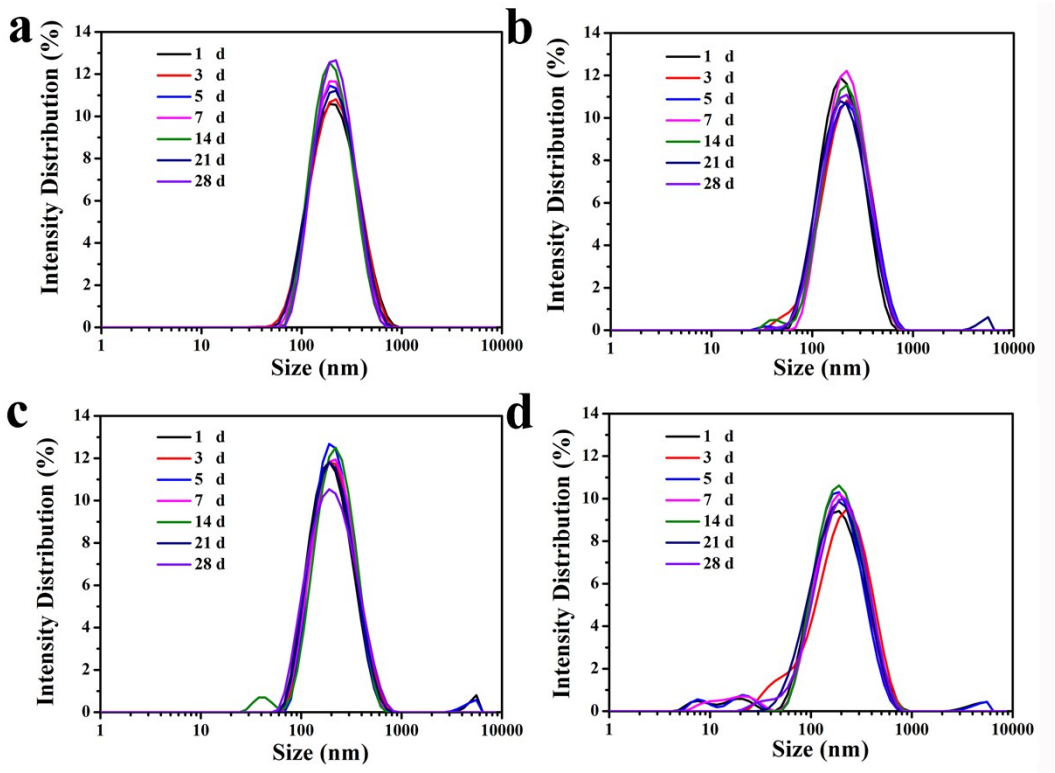

Fig. S5 Size distributions of DPP-IID-FA nanoparticles in (a) water, (b) PBS, (c) DMEM and (c) 10% FBS measured by DLS.

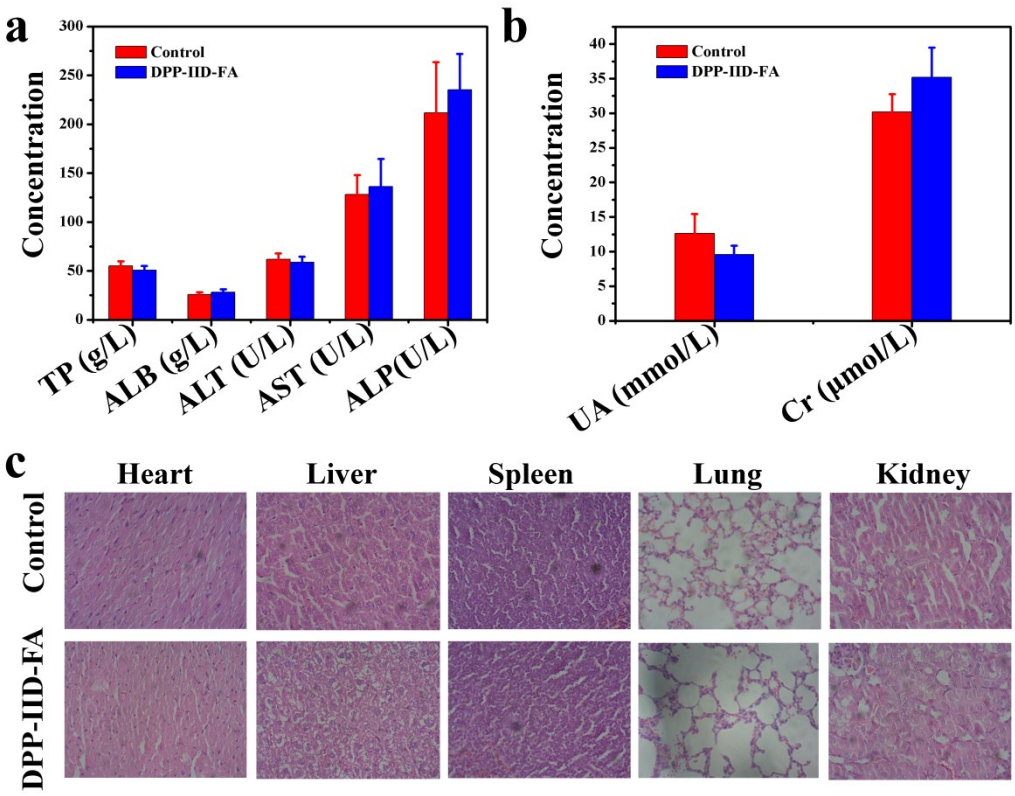

Fig. S6 Serum biochemical indicators obtained from mice at 7 days after injecting with DPP-IID-FA nanoparticles (n=5), or PBS solution (n=5, control) (A); hematoxylin and eosin (H&E)-stained tissue sections from mice at 7 days after injecting with DPP-IID-FA nanoparticles (n=5), or PBS solution (n=5, control).

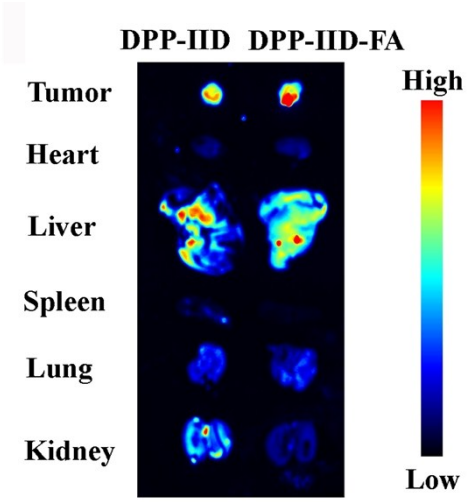

Fig. S7 *Ex vivo* fluorescence images of major organs obtained from mice after 24 h of indicated nanoparticle injection.

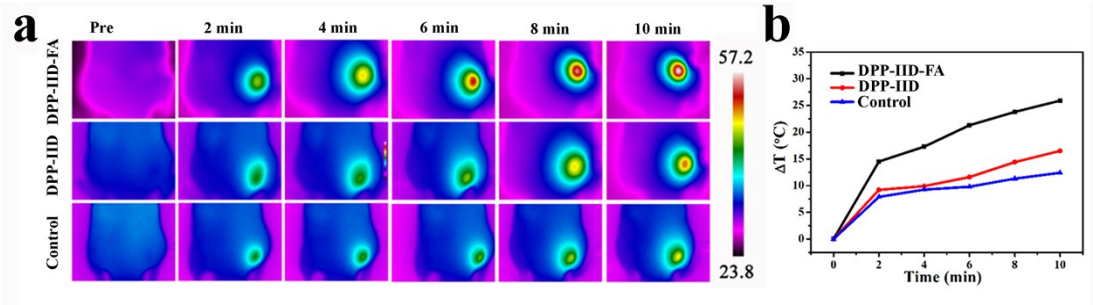

Fig. S8 (a) Infrared thermal images of Hela tumor-bearing mice injected with PBS, DPP-IID-FA or DPP-IID nanoparticles, then exposed to continues 1064 nm laser irradiation for 10 min at the power density of 1 $W \cdot cm^{-2}$. (b) Temperature elevation curve at the tumor site upon laser irradiation during the indicated treatment.

References

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