Supporting Information

Ultra-homogeneous NIR-II fluorescent self-assembled nanoprobe with AIE properties for photothermal therapy of prostate cancer

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Experimental Procedures

Materials

NMR spectra were recorded on a Bruker BioSpin (^{1}H 400 MHz, ^{13}C 100 MHz) spectrometer, and chemical shifts were reported on a delta scale in ppm relative to CDC_{l3} (δ = 7.26 ppm) for ^{1}H NMR and $CDCl_3$ (δ = 77.0 ppm) for ^{13}C NMR. Data were reported as follows: chemical shift; multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad signal); coupling constant (Hz); and integration. High resolution mass spectrometry (HR-MS) analyses were carried out using MALDI-TOF-MS. Flash chromatography was performed on silica gel (200~300 mesh). UV-vis absorption spectra of the NIR-II nanoprobe were recorded on a UNIC-3802 spectrophotometer. All chemicals were obtained from commercial suppliers (Innochem or Acros) and used without further purification. All air-sensitive reactions were carried out under nitrogen or argon.

Synthesis of 2, 6–dibromo-1,4,5,8-naphthalene tetracarboxylic dianhydride (2)

First, 1,4,5,8-naphthalene tetracarboxylic dianhydride **1** (5.0 g, 18.64 mmol) and a catalytic amount of iodine were added to a 250-mL flask containing 40 mL oleum. Next, bromine (2.05 mL, 37.34 mmol) was added slowly using an addition funnel at room temperature. The mixture was then heated at 50°C for 48 h while stirring. After cooling down to room temperature, excess bromine was removed by slowly adding a saturated solution of sodium thiosulfate. The residue was then carefully poured into ice water, which resulted in a yellow precipitate. The solids were then vacuum filtered and washed with copious amounts of water and dried under vacuum to obtain a bright yellow solid (6.3 g), which was used without further purification.

Synthesis of *N, N'*-(2'-ethyl) hexyl-2,6–dibromo-1,4,5,8-naphthalene

tetracarboxylic diimide (3)

First, 2-ethylhexyl amine (5.8 mL, 35.45 mmol) was added to a suspension of 2,6-dibromo-1,4,5,8-naphthalene tetracarboxylic dianhydride **2** (3.0 g, 7.04 mmol) in glacial acetic acid (60 mL) at room temperature using a syringe. The mixture was then refluxed for 2 h. After cooling down to room temperature, water (200 mL) was added to obtain the precipitate. The residue was purified via silica gel column chromatography (PE/CH_2Cl_2 = 4/1, *v/v*) to obtain the *N, N'*-(2'-ethyl) hexyl-2,6–dibromo-1,4,5,8-naphthalene tetracarboxylic diimide **3** as a pale yellow solid (1.5 g, 33%). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.00 (s, 2H), 4.20-4.11 (m, 4H), 1.98-1.90 (m, 2H), 1.42-1.26 (m, 16H), 0.96-0.86 (m, 12H).

Synthesis of 4,9-bis(4-(diphenylamino)phenyl)-2,7-bis(2-ethylhexyl)benzo[lmn] [3,8] phenanthroline-1,3,6,8(2H,7H)-tetraone (5)

Thirty milliliters of toluene and 5 mL aqueous solution were added to a mixture of *N, N'*-(2'-ethyl) hexyl-2, 6–dibromo-1,4,5,8-naphthalene tetracarboxylic diimide **3** (0.5 g, 0.77 mmol), *N, N*-dipheny l-4-(4, 4, 5, 5–tetramethyl-1,3,2-dioxaborolan–2-yl) aniline **4** (0.63 g, 1.70 mmol), a catalytic amount of Aliquat 336, K_2CO_3 (0.55 g, 3.98 mmol), and $Pd(PPh_3)_4$ (46 mg, 0.04 mmol). The mixture was degassed for 20 min, then heated at 120°C for 24 h under an argon atmosphere. After cooling down to room temperature, water was added, and the mixture was extracted with DCM. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (PE/CH_2Cl_2 = 1/2, *v/v*) to give **5** as a blue solid (305 mg, 40.5 %). ^1H NMR ($CDCl_3$, 400 MHz): δ 8.68 (s, 2H), 7.35-7.29 (m, 12H), 7.23 (dd, *J* = 8.5, 1.0 Hz, 8H), 7.16 (d, *J* = 8.7 Hz, 4H), 7.09 (t, *J* = 7.3 Hz, 4H), 4.15-4.02 (m, 4H), 1.95-1.86 (m, 2H), 1.37-1.25 (m, 16H), 0.94-0.85 (m, 12H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.63, 14.12, 23.09, 23.96, 26.94, 28.56, 30.66, 37.80, 44.32, 76.73, 77.05, 77.36, 121.98, 122.39, 123.55, 125.21, 125.34, 127.31, 129.42, 129.68, 133.46, 136.21, 147.14, 147.36, 148.09, 162.92, 163.13. HR-MS (MALDI-TOF) *m/z* calcd. for $C_{66}H_{64}N_4O_4$ $[M]^+$: 976.4928, Found 976.4874.

Nanofabrication of the NIR-II fluorescent molecules

Ten microliters of dichloromethane containing 2 mg fluorescent molecule was added to 5 mL deionized water (containing 5 mL PEG). After subjection to 90 s of ultrasonic treatment to ensure complete suspension, the preparation was repeatedly extruded 6 times with a liposome extruder (polycarbonate membrane with 200 nm pore size), until the nano-sized NIR-II fluorescent probe was obtained.

Characterization of the nanoprobe

The morphology and size of nanoprobe were observed using a transmission electron

microscope and a frozen transmission electron microscope, respectively. UV−vis absorbance measurements were carried out on a JASCO V-550 UV−vis spectrophotometer. Fluorescence measurements were carried out on a Jasco FP-6500 spectrofluorometer. DIS measurements were carried out on a Malvern Nano ZS-90. Temperature was measured using a hand-held near-infrared thermometer (Testo).

Biocompatibility evaluation of the nanoprobes

PC-3 cells were seeded in three 96-well plates. When the cells reached 60% confluence, nanoprobes with a concentration ranging between 0-160 μg mL^{-1} (calculated by the concentration of NIR-II fluorescent molecules) were added. After 24, 48, and 72 h incubation, cell proliferation rate was detected by performing CCK-8 assay using a kit according to the manufacturer's instruction.

Cell uptake of the nanoprobe

PC-3 cells were seeded in a 12-well plate with a pre-placed cover glass. When the cells reached 50% confluence, the nanoprobe with a concentration of 80 μg mL^{-1} (calculated by the concentration of NIR-II fluorescent molecules) was added. After 1, 12, and 24 h of incubation, the distribution and intensity of two-photon fluorescence were observed under a two-photon fluorescence microscope (excitation = 710 nm, emission = 400-631 nm).

In vitro PTT of the nanoprobe

PC-3 cells were seeded in a 6-well and a 24-well plate. After reaching 60% confluence, the cells were randomly divided into PBS, PBS + laser, nanoprobe, nanoprobe + laser groups, and subjected to different treatments according to the group; nanoprobe at 80 μg mL^{-1} was used for all groups. The cells were incubated for 12 h and then treated with or without 633 nm laser (0.8 W cm^2). Subsequently, flow cytometry analysis and a live/dead cell double staining assay were performed to evaluate the therapeutic effect.

In vivo distribution and metabolism of the nanoprobe

In this work, all animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Shanghai Jiao Tong University and approved by the Animal Ethics Committee of Shanghai Jiao Tong University (No. 202003010). First, 100 μL PC-3 cell suspension in saline (1×10^5 cells) was injected subcutaneously into the hind limbs of nude mice and mice used for subsequent experiments when the tumor volume reached approximately 200 mm^2. After intravenously injecting nanoprobe suspension (80 μL) into the tumor bearing mice, the distribution of fluorescence was observed and photographed under a living animal NIR-II fluorescence imager at 0, 0.5, 12, and 48 h, respectively.

In vivo PTT of the nanoprobe

One hundred microliters of the PC-3 cell suspension (1×10^5 cells) in saline was injected subcutaneously into the hind limbs of nude mice and used for *in vivo* PTT experiments when the tumor volume reached approximately 200 mm^2. PC-3 tumor bearing mice were randomly divided into 4 groups (n = 6): normal saline; normal saline + laser; nanoprobe; and nanoprobe + laser, and injected with normal saline and nanoprobe via intravenous injection. At 24 h post injection, tumor-bearing mice were treated with or without 633 nm laser irradiation for 8 min (0.8 W cm^2). During laser irradiation, the temperature of the tumor areas was monitored by a hand-held near-infrared imager. The tumor volume and weight of tumor bearing mice were measured on the 21st day following treatment. After observation, the main organs and tumor tissues of each group were sectioned, and Ki-67 immunostaining was performed.

Figures and legends

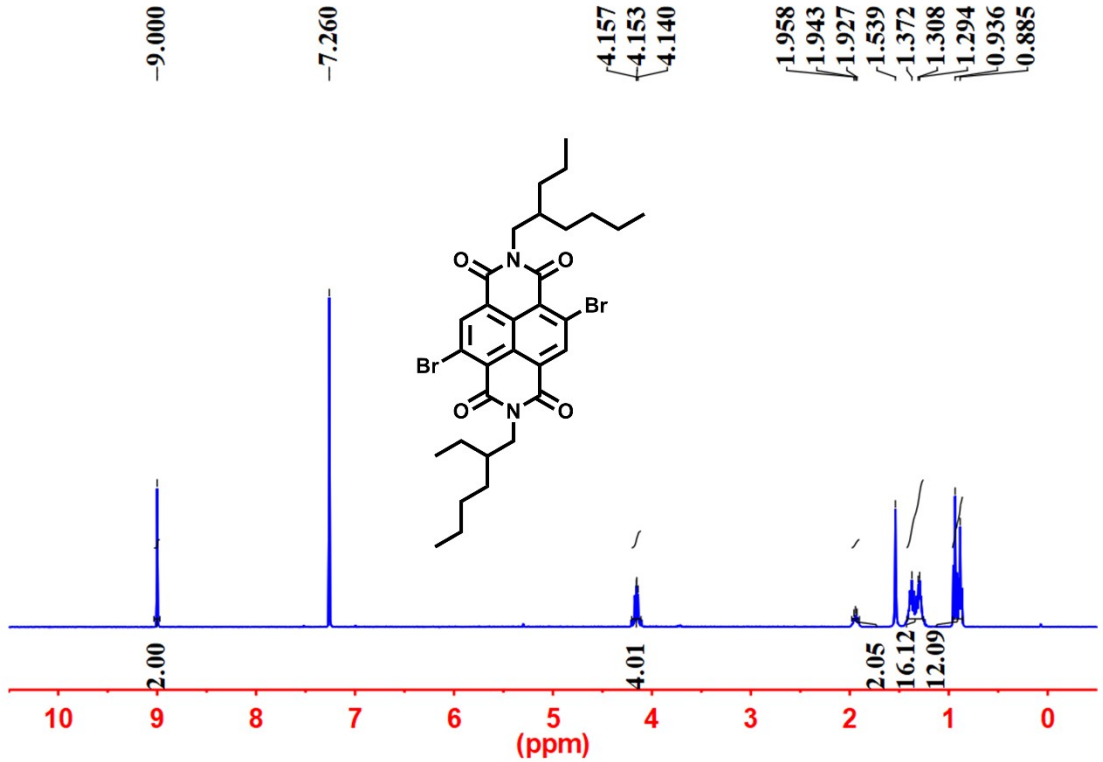

Figure S1. ^{1}H NMR spectrum of *N, N'*- (2'-ethyl) hexyl - 2, 6 – dibromo - 1, 4, 5, 8-naphthalene tetracarboxylic diimide **3** in $CDCl_3$.

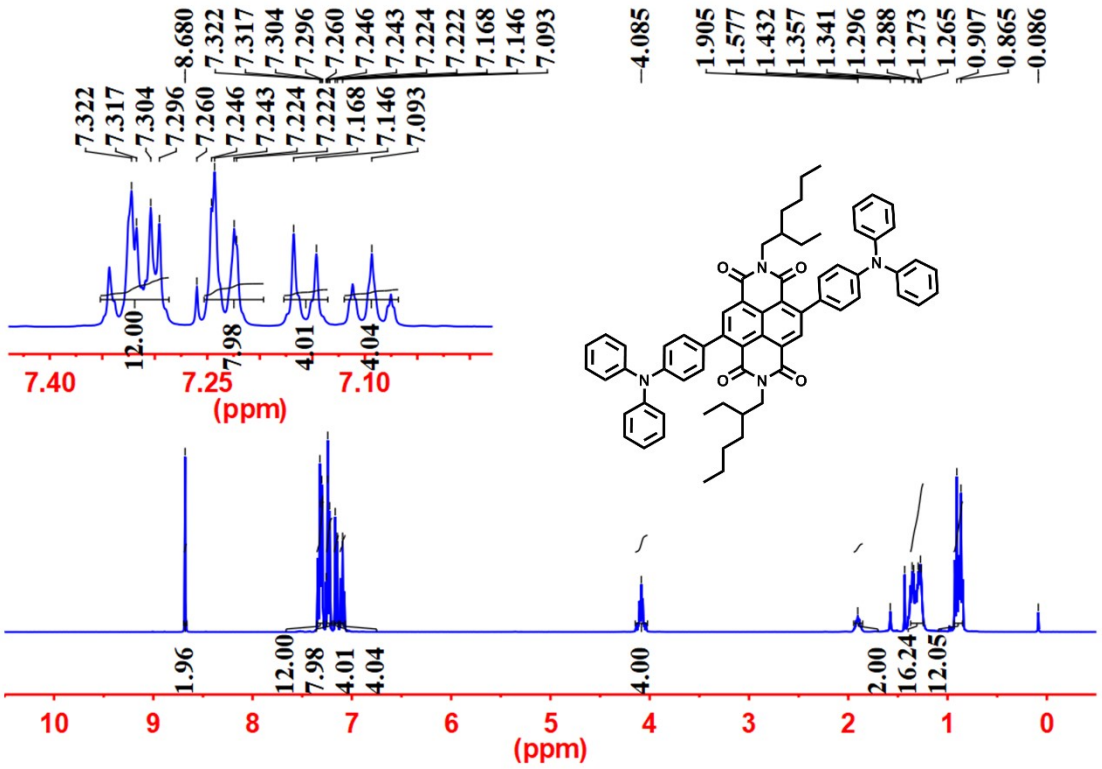

Figure S2. ^{1}H NMR spectrum of 4, 9 – bis (4- (diphenylamino) phenyl) - 2, 7 – bis (2-ethylhexyl) benzo [*lmn*] [3,8] phenanthroline - 1, 3, 6, 8 (*2H,7H*) - tetraone **5** in $CDCl_3$.

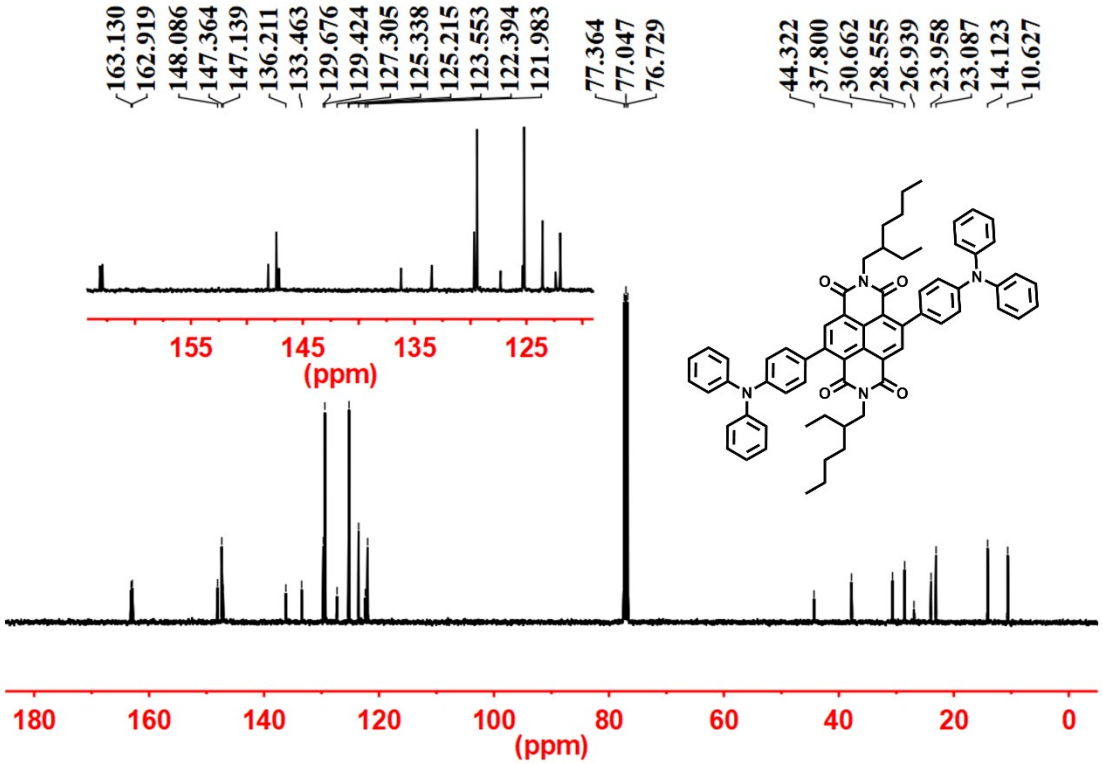

Figure S3. ^{13}C NMR spectrum of 4, 9- bis (4- (diphenylamino) phenyl) - 2, 7 – bis (2-ethylhexyl) benzo [*lmn*] [3,8] phenanthroline - 1, 3, 6, 8 (*2H,7H*) - tetraone **5** in $CDCl_3$.

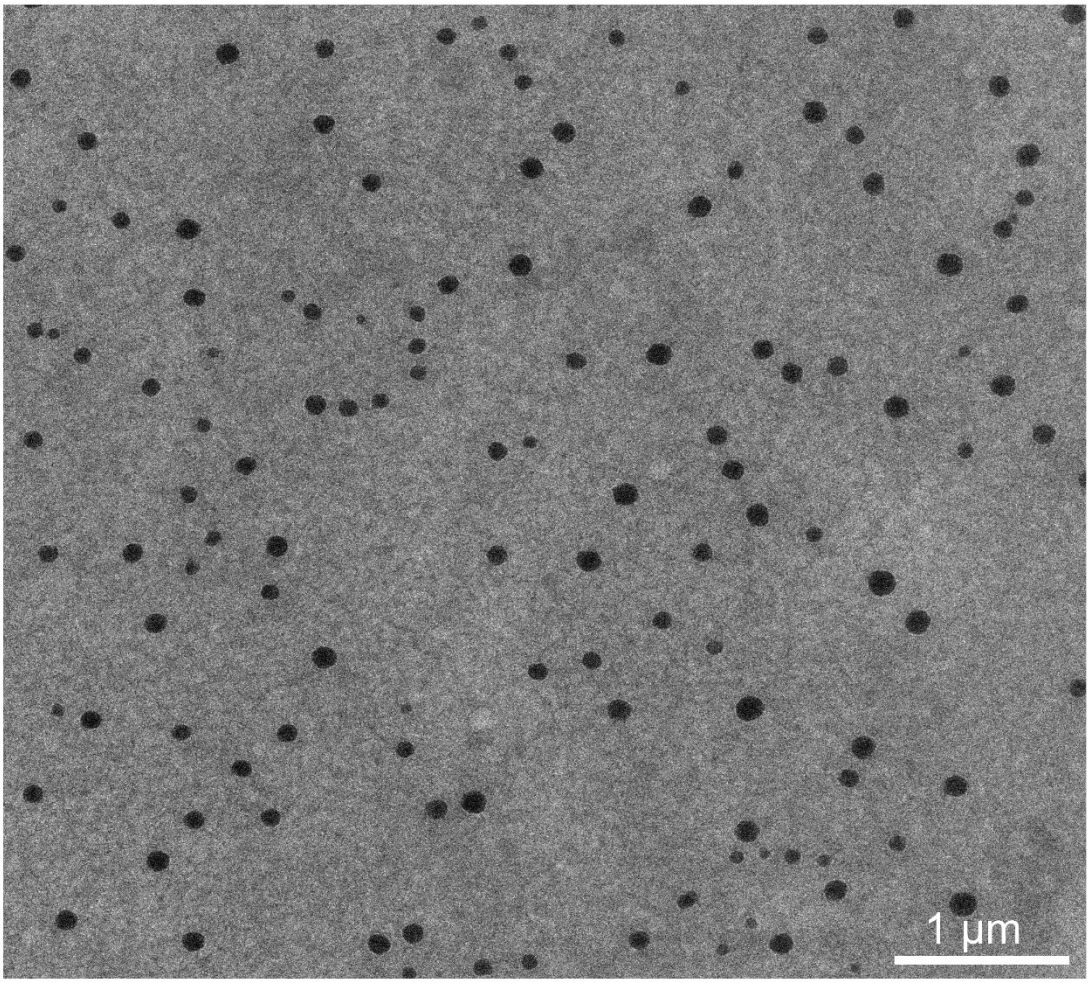

Figure S4. The TEM image of the NIR-II nanoprobes.

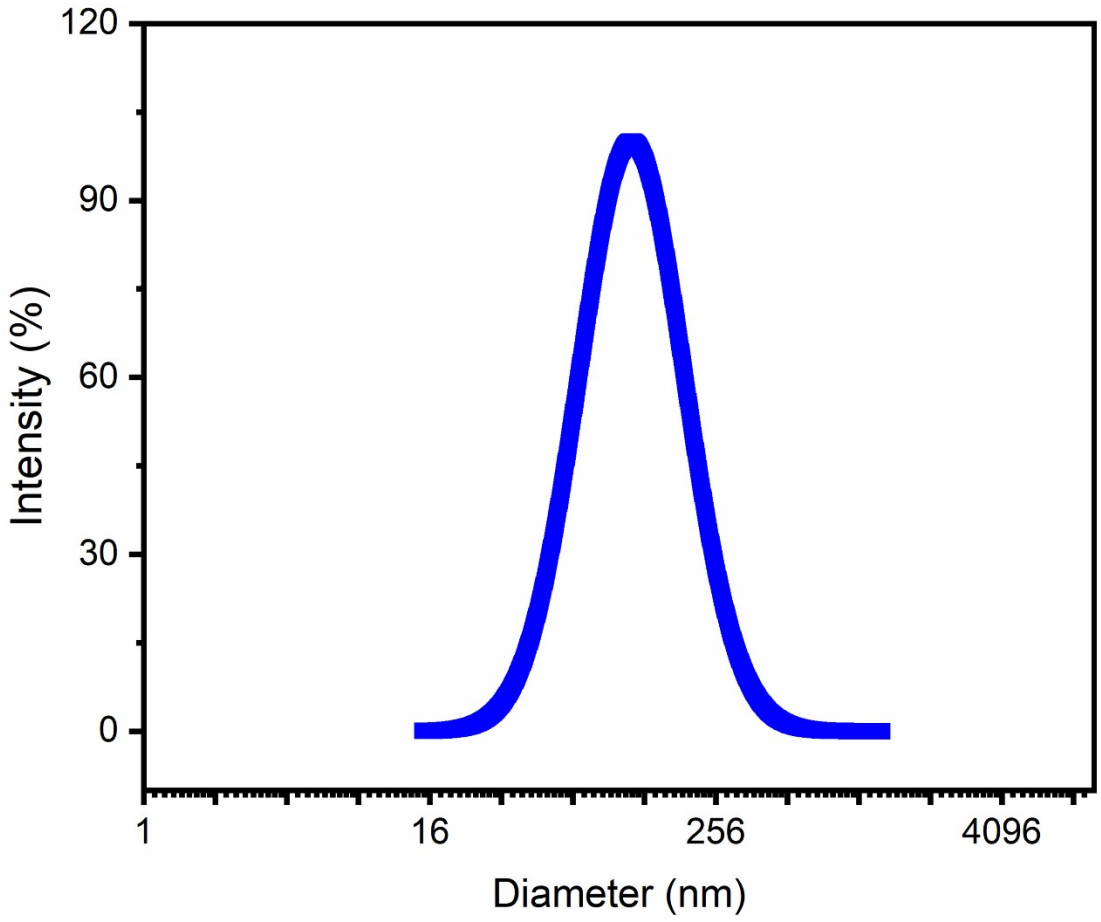

Figure S5. Hydration radius of the NIR-II nanoprobe in deionized water. Mean diameter = 138.6 nm.

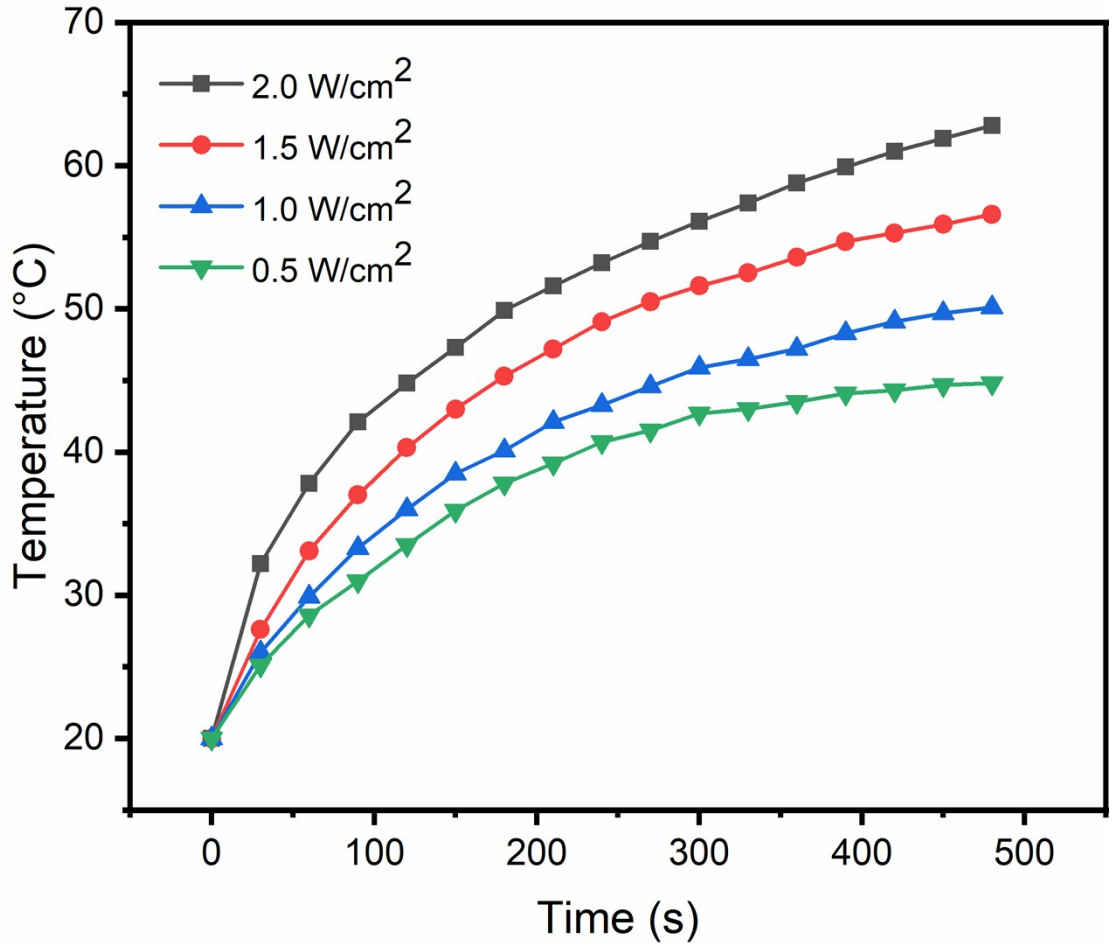

Figure S6. Photothermal performance of the NIR-II nanoprobes under different powers of 633 nm laser irradiation.

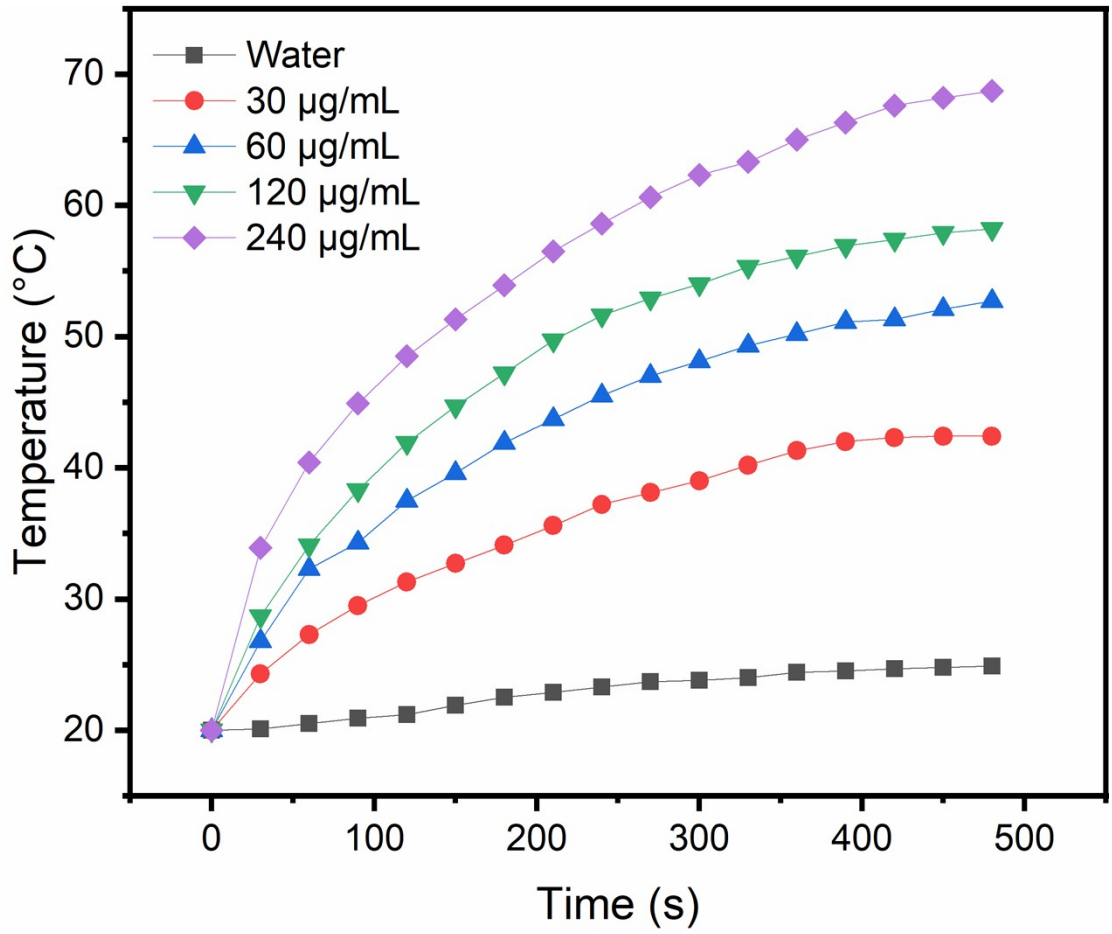

Figure S7. Photothermal performance of the NIR-II nanoprobes with different concentrations under 633 nm laser irradiation.

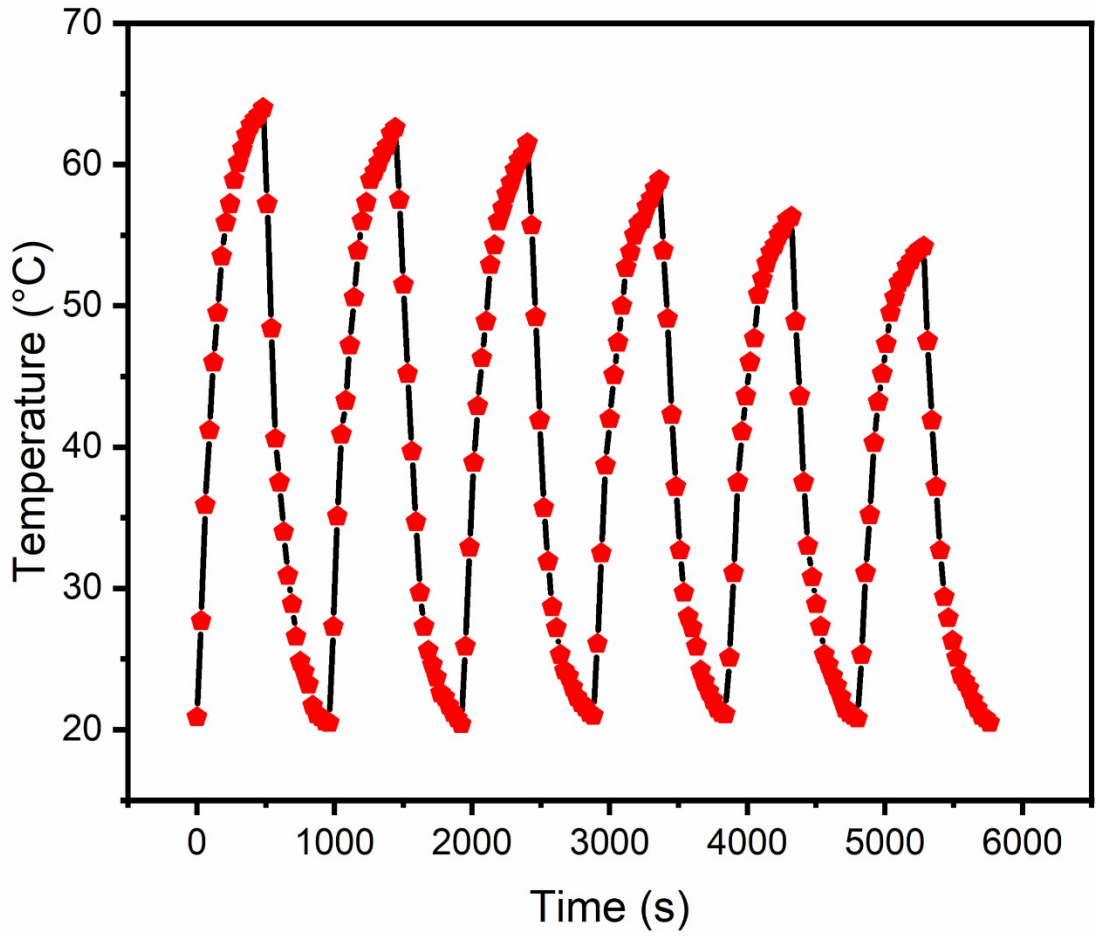

Figure S8. Heat-cold cycle experiment of the NIR-II nanoprobe under 633 nm laser irradiation.

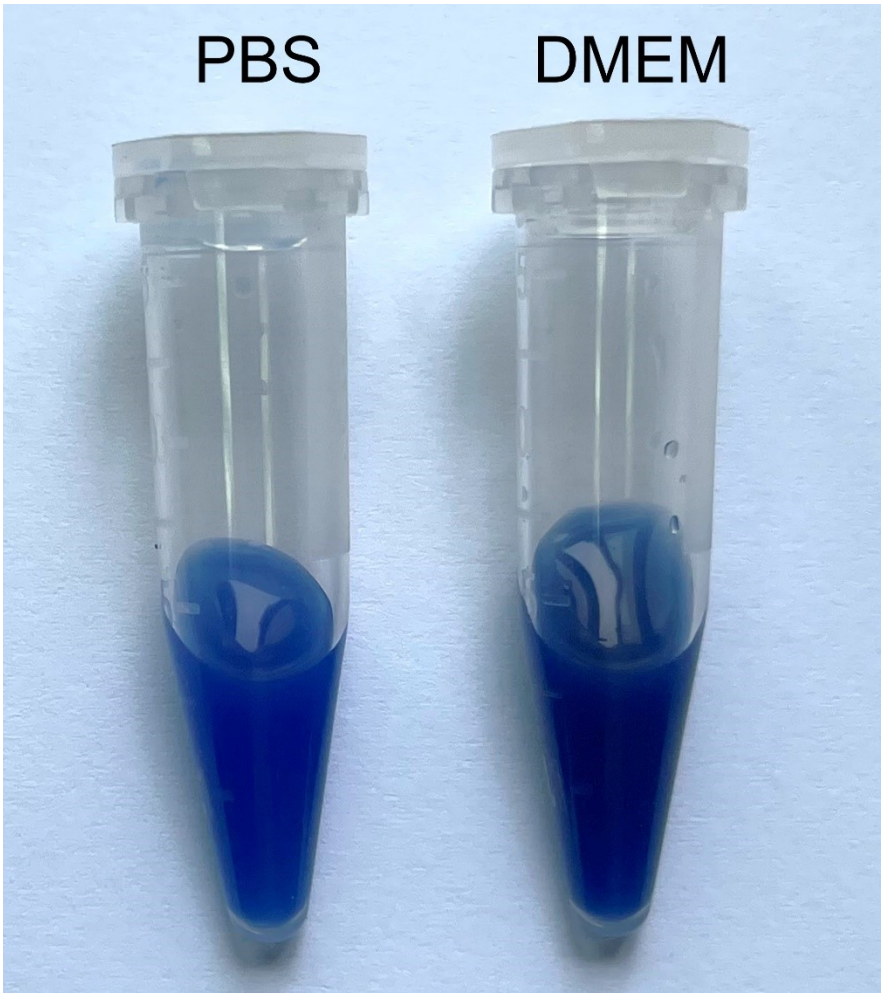

Figure S9. Photograph of nanoprobes suspended in PBS or DMEM containing10% FBS for 28 d.

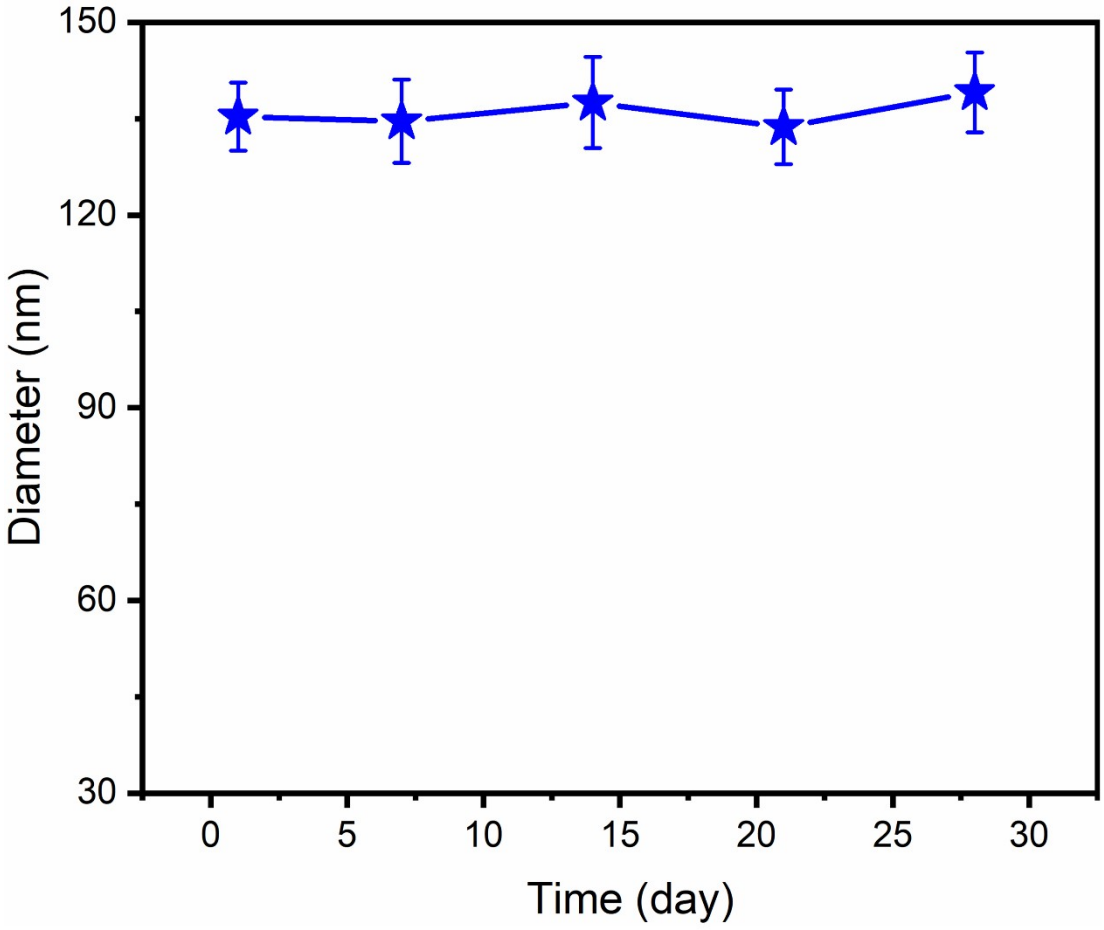

Figure S10. Changes in the hydration radius of the nanoprobe placed in PBS for 28 d.

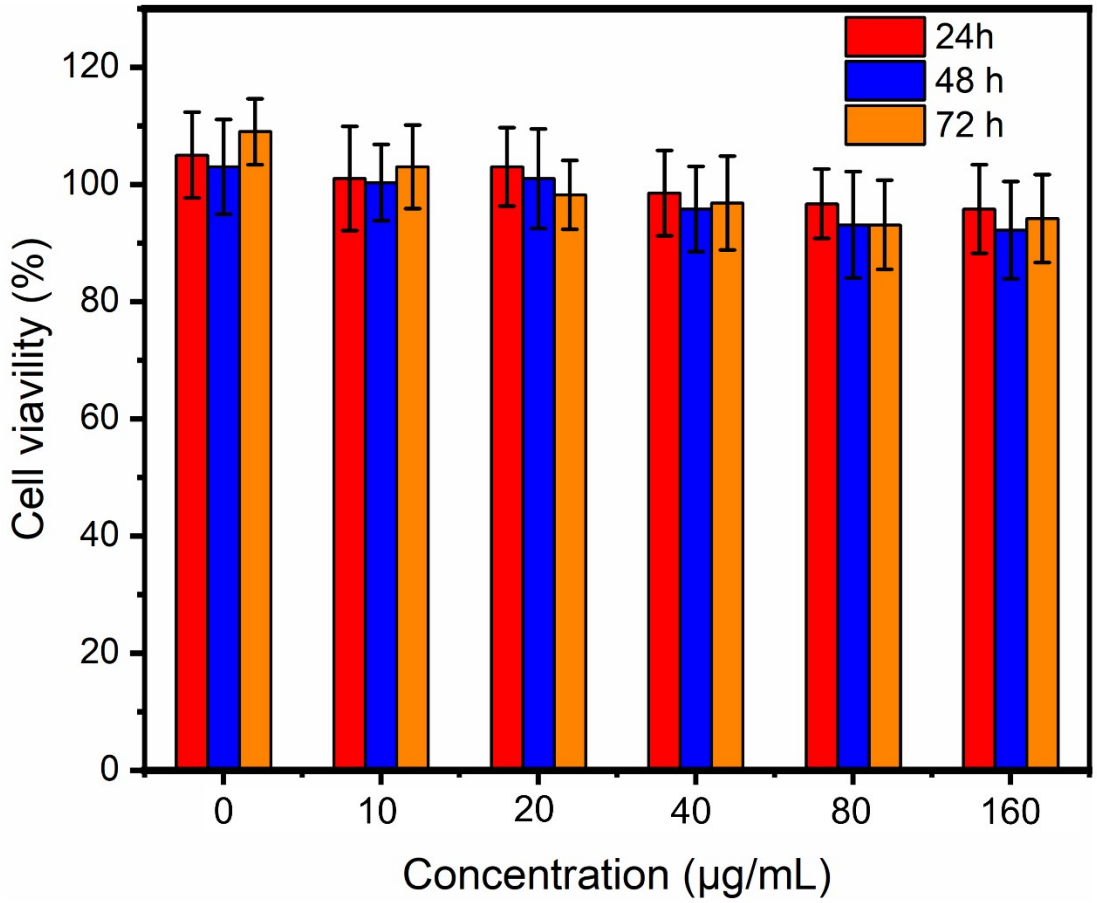

Figure S11. The effect of nanoprobes with a concentration range of 0-160 μg/mL on cell proliferation following incubation with cells for 24, 48, and 72 h, respectively.

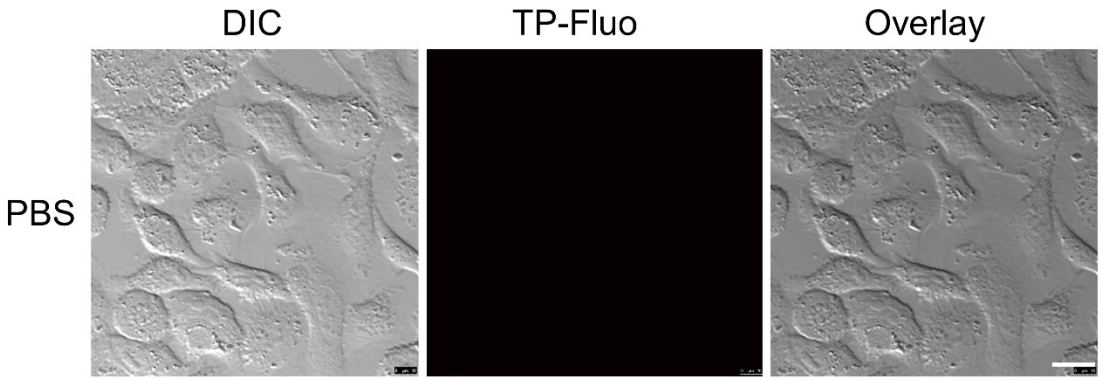

Figure S12. Two-photon fluorescence images of PC-3 cells and PBS following incubation for 12 h, (excitation = 710 nm, emission = 400-631 nm). Scale bar: 20 μm.