Electronic Supplementary Material (ESI) for Materials Chemistry Frontiers.

Supporting Information

Single Laser Activated Photodynamic/Photothermal Cancer Therapy Using Single Mitochondrial-Targeted Phototherapeutic Agent with Aggregation-Induced Emission Characteristic

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1. Experimental Section

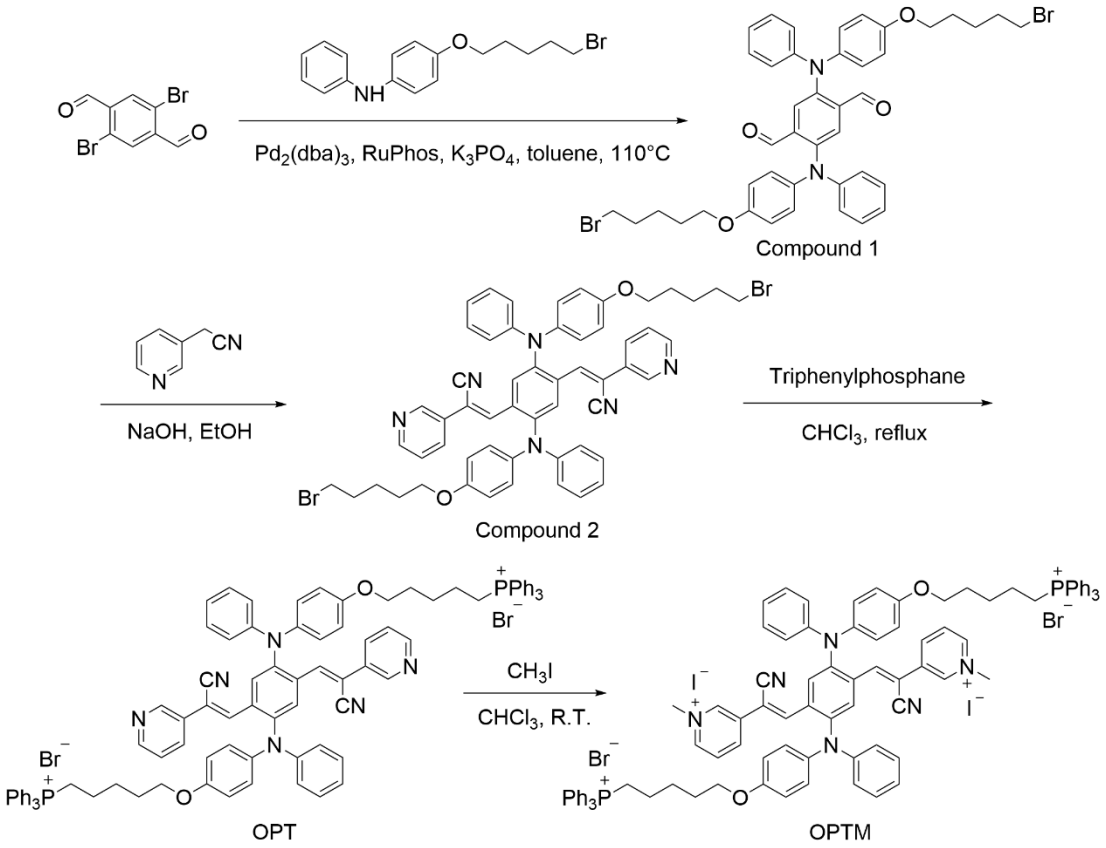
1.1 Materials

Iodomethane, 3-pyridylacetonitrile, 2,5-dibromoterephthalaldehyde, 4-hydroxydiphenylamine, Ruphos were purchased from Energy Chemical. Triphenylphosphine, K_2CO_3, $Pd_2(dba)_3$ and 1,5-dibromopentane were purchased from Heowns. Fetal bovine serum (FBS), phosphate buffered saline (PBS, pH = 7.4), RPMI-1640 culture medium, penicillin and streptomycin were purchased from Gibco. Mito-Tracker Green, 4′,6-diamidino-2-phenylindole (DAPI), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) and Cell Counting Kit-8 (CCK-8) were purchased from Beyotime Biotechnology. All chemicals and reagents were commercially available and used directly without further purification.

1.2 Instruments

^{1}H NMR spectra were recorded with a Bruker AV-400 (400 MHz) spectrometer at room temperature with tetramethylsilane (TMS, δ = 0 ppm) as an internal standard. ^{13}C NMR spectra were determined on a Bruker AV-400 (100 MHz) spectrometer with tetramethylsilane (TMS, δ = 0 ppm) as an internal standard. High resolution mass spectra (HRMS) were performed on a Thermo Q-Exactive Focus MS with an ESI source. UV-vis spectra were recorded on a Shimadzu UV-3390 spectrophotometer. Fluorescence spectra were carried out with an Edinburgh FLS1000 spectrometer. Dynamic light scattering (DLS) measurement was performed using a Malvern Nano-ZS instrument at room temperature. The morphology of nanoparticles (NPs) was observed using a Zeiss MERLIN Compact type field emission scanning electron microscope (SEM).

1.3 Synthesis of OPT and OPTM



Scheme S1. Synthetic routes of OPT and OPTM.

Synthesis of compound 1: A mixture of 1,5-dibromopentane (2.300 g, 10.00 mmol) and K_2CO_3 (1.040 g, 7.50 mmol) in DMF (3 mL) was stirred for 10 min. 4-Hydroxydiphenylamine (0.926 g, 5.00 mmol) in DMF (3 mL) was added dropwise slowly. After dropwise, the solution was stirred at room temperature for 2 h and then reacted at 80°C for 2 h. After cooling to room temperature, the mixture was poured into water and extracted with dichloromethane. The residue was purified by silica gel chromatography eluted with petroleum ether/dichloromethane (v/v = 2:1) to give a white solid product. A mixture of the above product (1.337 g, 4.00 mmol), 2,5-dibromoterephthalaldehyde (0.234 g, 0.80 mmol), K_3PO_4 (1.696 g, 8.00 mmol), Ruphos (0.112 g, 0.24 mmol) and $Pd_2(dba)_3$ (0.055 g, 0.06 mmol) in toluene (4 mL) was refluxed at 110°C for 12 h under nitrogen. The resulting mixture was diluted with dichloromethane and washed with water. The organic phase was separated, dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure and purified by silica gel chromatography eluted with petroleum ether/dichloromethane (v/v = 1:1) to give compound 1 (60% yield). ^{1}H NMR (400 MHz, DMSO-

d$_6$, TMS, ppm): δ 10.02 (s, 2H), 7.31-7.25 (m, 6H), 7.13-7.10 (m, 4H), 6.99-6.93 (m, 10H), 3.97-3.94 (t, *J* = 6.4 Hz, 4H), 3.59-3.55 (t, *J* = 6.4 Hz, 4H), 1.91-1.83 (m, 4H), 1.77-1.70 (m, 4H), 1.58-1.50 (m, 4H); ^{13}C NMR (100 MHz, DMSO-*d*$_6$, TMS, ppm): δ 190.2, 156.0, 149.7, 146.2, 141.0, 135.7, 130.1, 127.6, 126.9, 122.6, 121.4, 116.2, 67.9, 35.6, 32.4, 28.3, 24.8.

Synthesis of compound 2: A mixture of 3-pyridylacetonitrile (0.047 g, 0.40 mmol) and compound 1 (0.160 g, 0.20 mmol) was stirred in ethanol (10 mL) catalyzed by dozens of drops of NaOH solution (0.25 M) at room temperature for 1 h. The residue was obtained by filtration, washed with ethanol and water respectively, and dried to give compound 2 (91% yield). ^{1}H NMR (400 MHz, $CDCl_3$, TMS, ppm): δ 8.55-8.54 (dd, *J* = 4.8, 1.2 Hz, 2H), 8.41 (d, *J* = 2.4 Hz, 2H), 7.72 (s, 2H), 7.56-7.51 (m, 4H), 7.29-7.24 (m, 6H), 7.12-7.10 (d, *J* = 8.8 Hz, 4H), 7.04-7.03 (d, *J* = 8.0 Hz, 4H), 6.98-6.96 (t, *J* = 7.2 Hz, 2H), 6.86-6.84 (d, *J* = 9.2 Hz, 4H), 3.94-3.91 (t, *J* = 6.4 Hz, 4H), 3.47-3.44 (t, *J* = 6.4 Hz, 4H), 1.96-1.92 (m, 4H), 1.82-1.78 (m, 4H), 1.64-1.60 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$, TMS, ppm): δ 155.6, 150.0, 148.2, 146.8, 143.4, 141.2, 140.6 , 133.3, 132.4, 129.9, 129.5, 128.1, 126.1, 123.4, 122.3, 121.8, 116.1, 115.7, 111.1, 67.9, 33.7, 32.5, 28.4, 24.8.

Synthesis of OPT: A mixture of compound 2 (0.100 g, 0.10 mmol) and triphenylphosphine (0.262 g, 1.00 mmol) in chloroform (5 mL) was refluxed at 60°C. The end point of the reaction was monitored by TLC. After cooling to room temperature, the solvent was evaporated under reduced pressure. The residue was purified by silica gel chromatography eluted with dichloromethane/ethanol (v/v = 10:1) to give OPT (63% yield). ^{1}H NMR (400 MHz, $CDCl_3$, TMS, ppm): δ 8.53-8.52 (d, *J* = 4.4, Hz, 2H), 8.36 (s, 2H), 7.88-7.78 (m, 18H), 7.73-7.68 (m, 14H), 7.53-7.49 (m, 4H), 7.31-7.30 (m, 2H), 7.27-7.23 (d, *J* = 8.0 Hz, 4H), 7.07-7.01 (m, 8H), 6.95-6.92 (t, *J* = 7.2 Hz, 2H), 6.76-6.73 (d, *J* = 9.2 Hz, 4H), 3.87-3.84 (m, 8H), 1.85-1.72 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$, TMS, ppm): δ 155.5, 149.9, 148.1, 146.6, 143.3, 141.4, 140.5, 135.0, 133.7, 133.4, 132.4, 130.5, 129.9, 129.5, 128.0, 126.1, 123.7, 122.3, 121.7, 118.8, 117.9, 116.1, 115.7, 110.9, 67.9, 28.6, 27.0, 22.5, 22.4.

1.4 Detection of ROS generation in solution

DCF-DA was used as an indicator to detect the reactive oxygen species (ROS) generation of OPTM NPs under laser irradiation. Concisely, 0.25 mL of DCF-DA ethanol solution (1 mM) was added to 1 mL of NaOH aqueous solution (10 mM) and stirred for 0.5 h at room temperature, followed by the addition of 5 mL of 1 × PBS (pH = 7.4) to obtain DCF-DA solution (40 μM), and store refrigerated and protected from light. DCF-DA solution (50 μL, 40 μM) was mixed with 1

mL of OPTM NPs solution (0.5 mM). Then the mixed solution was irradiated with laser (650 nm, 0.06 W/cm^2) for different times intervals. The fluorescence change of the solution was measured with excitation at 488 nm and the emission was collected from 508 to 650 nm.

1.5 Calculation of photothermal conversion efficiency

The photothermal conversion efficiency (η) of OPTM NPs was calculated based on the Roper method.[1] OPTM NPs were irradiated with a 650 nm laser at a power density of 0.5 W/cm^2 for 5 min. Subsequently, the laser was turned off and cooled for 5 min. The photothermal conversion efficiency was calculated by the following equation.

$$\eta = \frac{hS(T_{max}-T_{sur})-Q_{dis}}{I(1-10^{-A_{650}})} \quad (1)$$

$$Q_{dis} = hS(T_{\max\,(water)} - T_{sur}) \quad (2)$$

where h and S represent the heat transfer coefficient and the surface area of the vessel, respectively. T_{max} and T_{sur} represent the maximum temperature and the surrounding temperature, respectively. Q_{dis} is the heat dissipation of water. I is the power density of the laser (0.5 W/cm^2), and A_{650} is the absorbance of OPTM NPs at 650 nm. The value of hS can be calculated by the following equation.

$$hS = \frac{m_D \cdot c_D}{\tau_s} \quad (3)$$

where m and c represent the mass (1.0 g) and heat capacity (4.2 J/g) of water. τ_s is the system time constant calculated by the following equation.

$$t = -\tau_s(ln\theta) \quad (4)$$

where θ is defined as the ratio of ΔT and ΔT_{max}, and θ can be obtained by the following equation.

$$\theta = \frac{T-T_{sur}}{T_{max}-T_{sur}} \quad (5)$$

1.6 Cell culture

4T1 cells and HeLa cells were cultured in RPMI-1640 medium, supplemented with 10% FBS, 1% penicillin and streptomycin, respectively. The cells were maintained in an atmosphere of 5% CO_2 and 95% humidified air at 37 °C. Cells were seeded and cultured in glass-bottom dishes for 24 h. Prior to experiments, the medium was removed and the adherent cells were washed twice with PBS buffer to remove the remnant growth medium.

1.7 Intracellular ROS detection

DCF-DA was used as a fluorescent probe to detect intracellular ROS generation. OPTM NPs (0.5 mM) were incubated with 4T1 cells to a final concentration of 100 μM for 2 h. Then, the cells were rinsed with PBS for three times and stained with 20 μL of DCF-DA at 37 °C for 10 min. Afterwards,

the cells were exposed to laser irradiation (650 nm, 0.3 W/cm^2, 5 min), followed by CLSM imaging. (DCF-DA: λ_{ex} = 488 nm, λ_{em} = 500-530 nm).

1.8 Cytotoxicity assay

The cytotoxicity and phototherapeutic capacity of OPTM NPs *in vitro* were assessed using the Cell Counting Kit-8 (CCK-8) method. For the dark toxicity of OPTM NPs, 4T1 cells were inoculated in 96-well plates at a cell density of 5000 cells/well and incubated with 1640 medium at 37°C for 24 h to adhere the cells to the wall. The above medium was replaced with OPTM NPs aqueous solution containing different concentrations (0 μM, 10 μM, 25 μM, 50 μM and 100 μM) and incubated for 12 h. The medium was discarded and washed three times with PBS buffer. Cells were then incubated with fresh medium containing 10% CCK-8 for 1 h in the dark. Finally, the absorbance of the solution at 450 nm was measured by a microplate reader (Epoch, BioTek, Gene company Limited). For the phototoxicity of OPTM NPs, referring to the dark toxicity method described above, the cells were first co-cultured with OPTM NPs for 6 h, and then laser irradiated (650 nm, 0.3 W/cm^2, 5 min) as following treatments: on ice for single PDT, with the addition of *N*-acetylcysteine (an effective ROS scavenger) for single PTT or laser irradiation without any treatment for simultaneous PDT/PTT. Subsequently, the cells were continued to incubate in dark for another 6 h. The cell viability in each well was calculated from the obtained values as a percentage of control wells. The results were presented as a mean and standard deviation obtained from four samples.

1.9 Live-Dead Cell Staining

4T1 cells were incubated with RPMI-1640 medium containing OPTM NPs (0.1 mM) at 37°C in 5% CO_2 for 6 h. After that, the cells were washed twice with PBS and then upon 650 nm laser irradiation (0.3 W/cm^2) for 0 or 5 min. Afterward, the cells were incubated with FDA for 10 min (10 μg/mL). The cells were incubated with PI in the dark (20 μg/mL) for another 10 min. Finally, cells were examined with CLMS. The green fluorescence from FDA was collected from 505 to 525 nm upon excitation at 488 nm. The red fluorescence from PI was collected from 570 to 610 nm upon excitation at 543 nm.

1.10 Cell apoptosis detection

4T1 cells were incubated with RPMI-1640 medium containing OPTM NPs (0.1 mM) at 37°C in 5% CO_2 for 6 h. After that, the cells were washed twice with PBS and then upon 650 nm laser irradiation (0.3 W/cm^2) for 0 or 5 min. After that, the cells were incubated at 4 ºC for another 0.5

h. Subsequently, the cells were washed with PBS and collected by centrifugation at 1000 rpm for 5 min at 4 ºC. The samples were then stained with an Annexin V-FITC/PI Apoptosis Detection Kit according to manufacturer's instructions and analyzed by FACS.

1.11 Tumor model establishment

All animal experiments followed the protocols approved by the Animal Ethics Committee of Institute of Radiological Medicine, Chinese Academy of Medical Sciences. The BALB/c female mice (5~6 weeks old) used in the experiments were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The tumor models were established by subcutaneous injection of 1 × 10^6 4T1 cells in PBS buffer into the right flanks of each mouse. After about a week, mice with tumor volumes at about 50 mm^3 were used subsequently.

1.12 *In vivo* photothermal imaging

4T1 tumor-bearing mice were intravenously injected with normal saline (100 μL) or OPTM NPs (100 μL, 0.5 mM). After 24 h, the tumor sites of the mice were irradiated with laser (650 nm, 0.5 W/cm^2), and the photothermal images and increasing temperatures were monitored in real time using an infrared thermal camera (HIKVISION H10). Mice injected with saline under the same irradiation conditions were used as the control.

1.13 Blood and serum biochemical parameters

Healthy female BALB/c mice were intravenously injected with saline or OPTM NPs. Blood parameters and serum biochemical (liver/kidney indicators) parameters of mice were systematically analyzed at 24 h post-injection. The standard hematology markers include white blood cells (WBC), red blood cells (RBC), platelets (PLT), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (HGB), mean corpuscular volume (MCV), mean platelet volume (MPV), mean corpuscular hemoglobin (MCH), lymphocytes (LY), hematocrit (HCT) and red blood cell width distribution (RDW). Serum biochemical analyses include aspartate transaminase (AST), alkaline phosphatase (ALP), alanine transaminase (ALT), blood urea nitrogen (BUN), uric acid (UA), creatinine (CR) and total bile acids (TBA).

1.14 H&E staining

After 15 days of treatment, the major organs of the mice (heart, liver, spleen, lung and kidney) were collected, fixed in 4% paraformaldehyde, then embedded in paraffin and cut into 4 μm thick sections. Sections were stained with hematoxylin and eosin (H&E) and imaged by an inverted optical microscopy.

2. Characterization of compounds

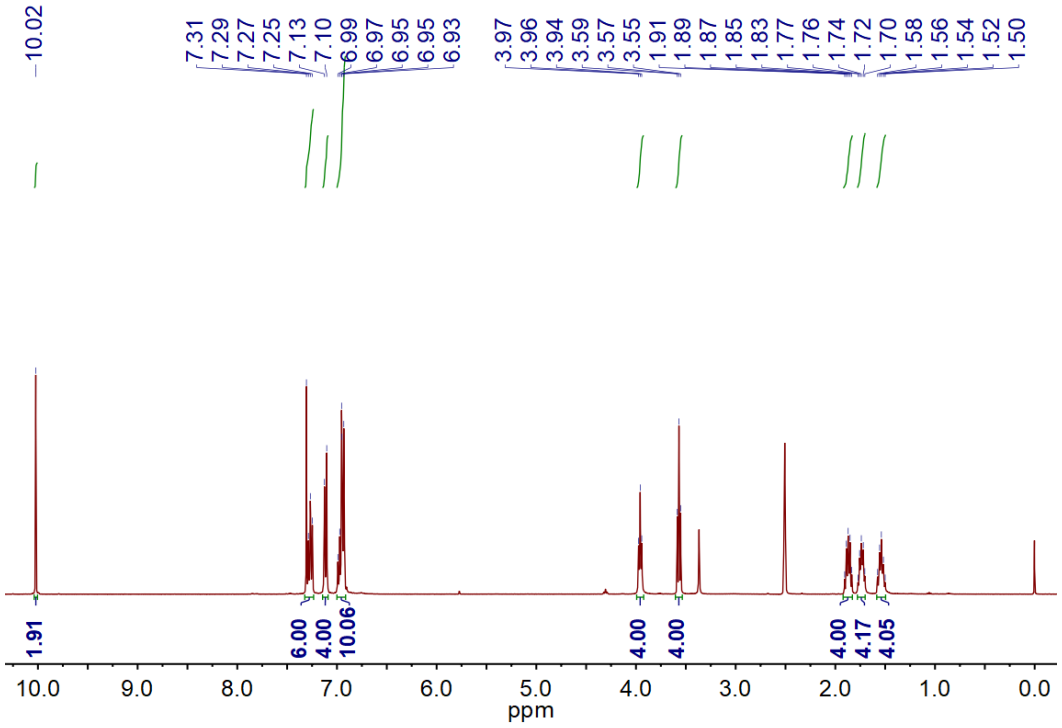


Figure S1. ^{1}H NMR spectrum of compound 1 in DMSO-d_6.

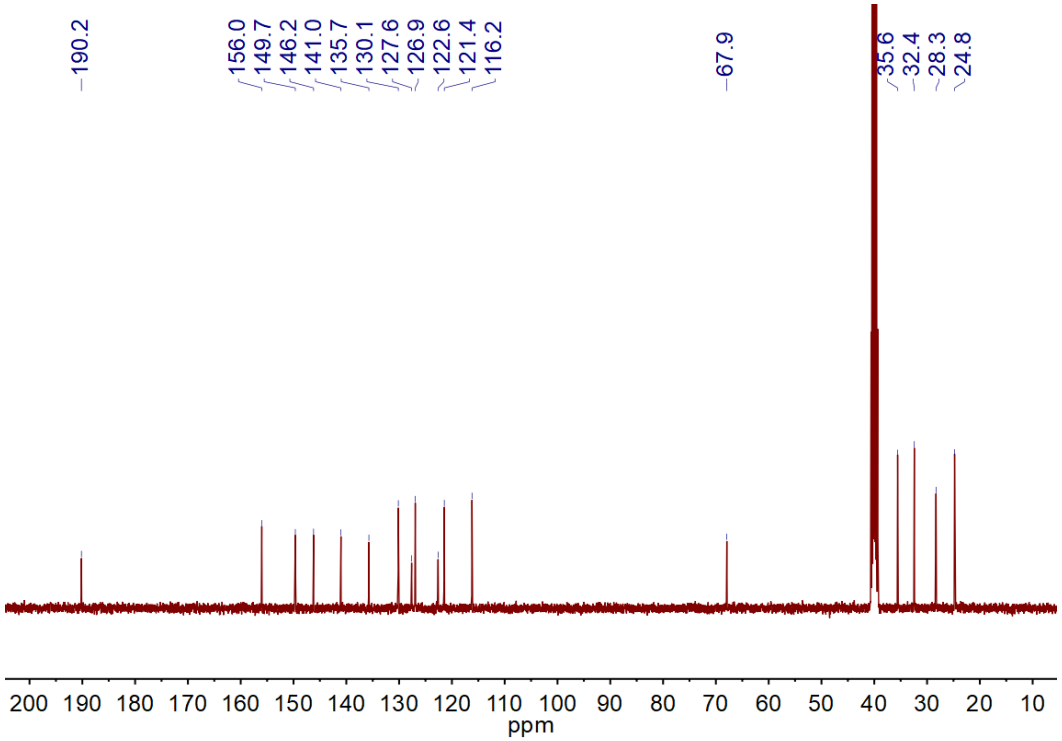


Figure S2. ^{13}C NMR spectrum of compound 1 in DMSO-d_6.

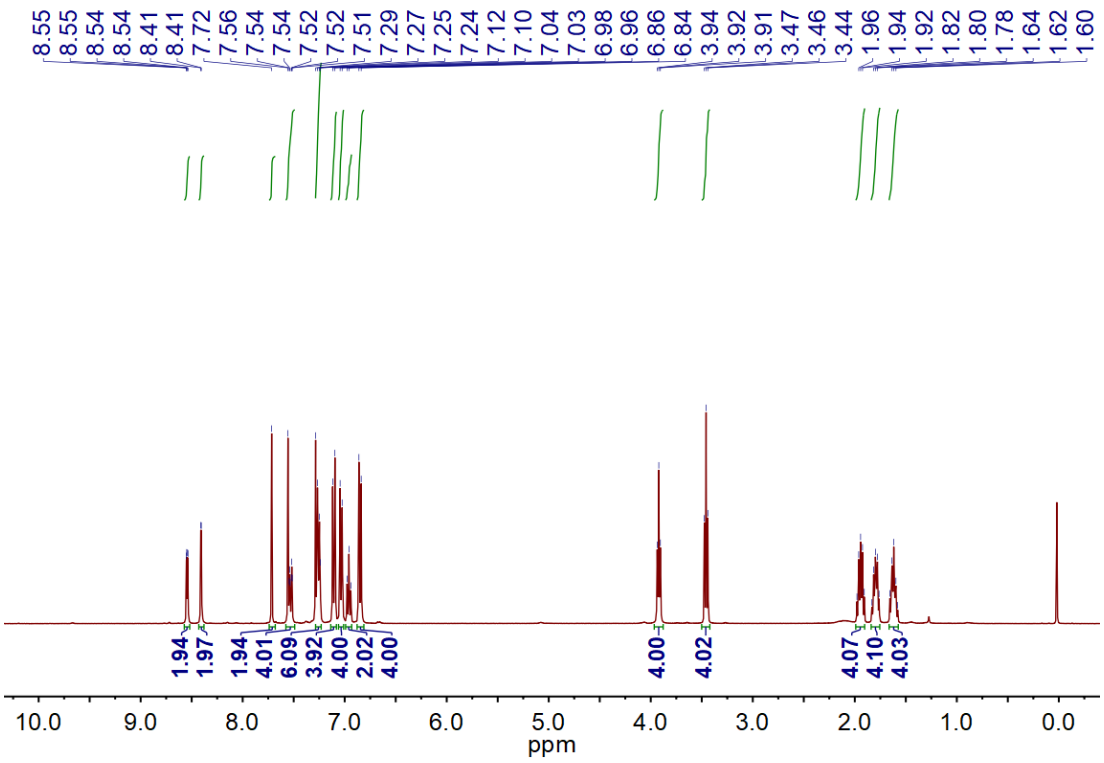


Figure S3. ^{1}H NMR spectrum of compound 2 in $CDCl_3$.

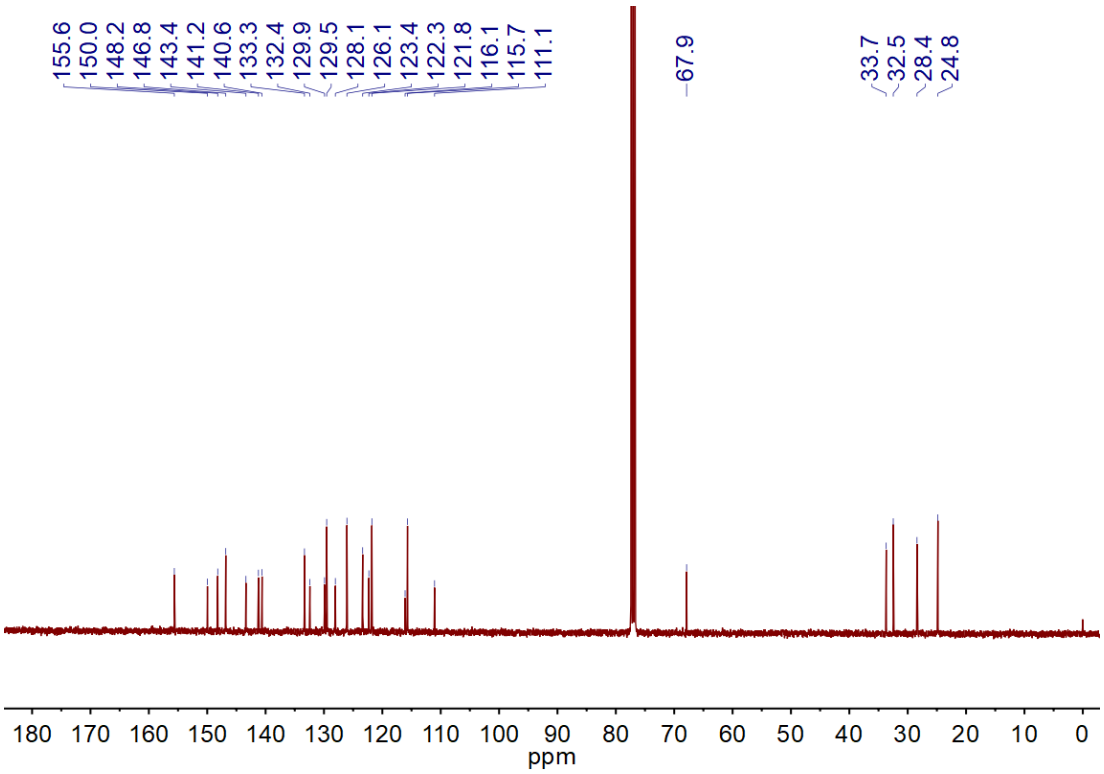


Figure S4. ^{13}C NMR spectrum of compound 2 in $CDCl_3$.

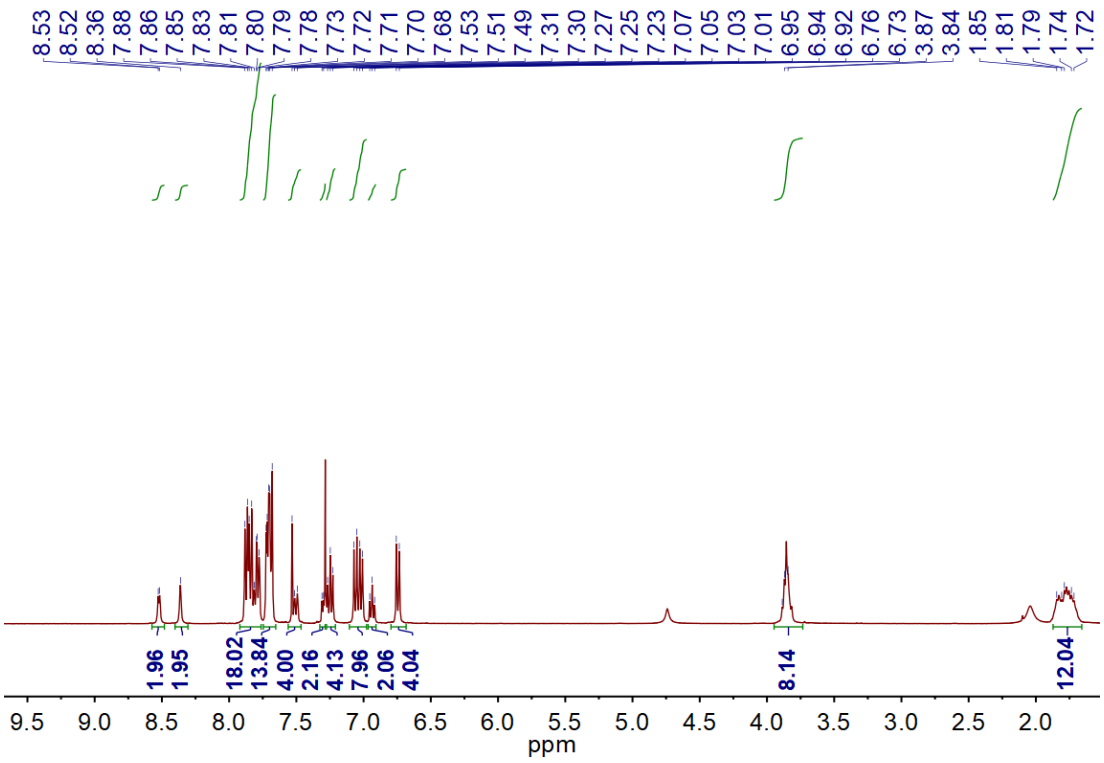


Figure S5. ^{1}H NMR spectrum of OPT in $CDCl_3$.

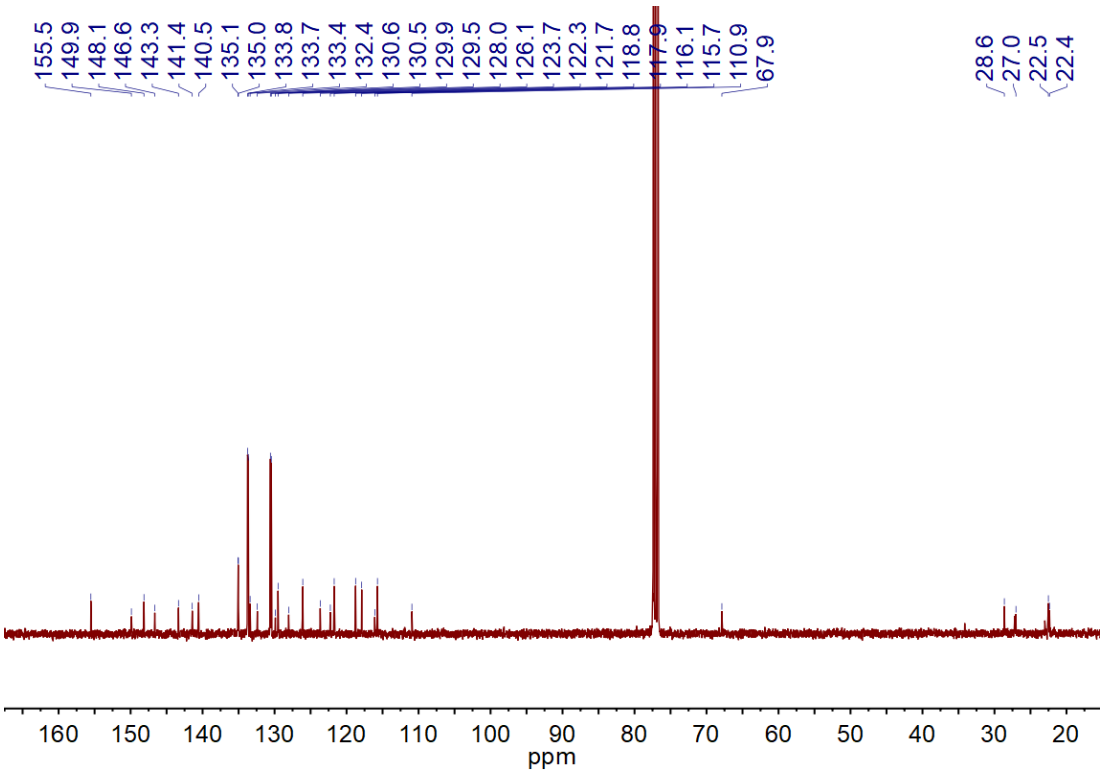


Figure S6. ^{13}C NMR spectrum of OPT in $CDCl_3$.

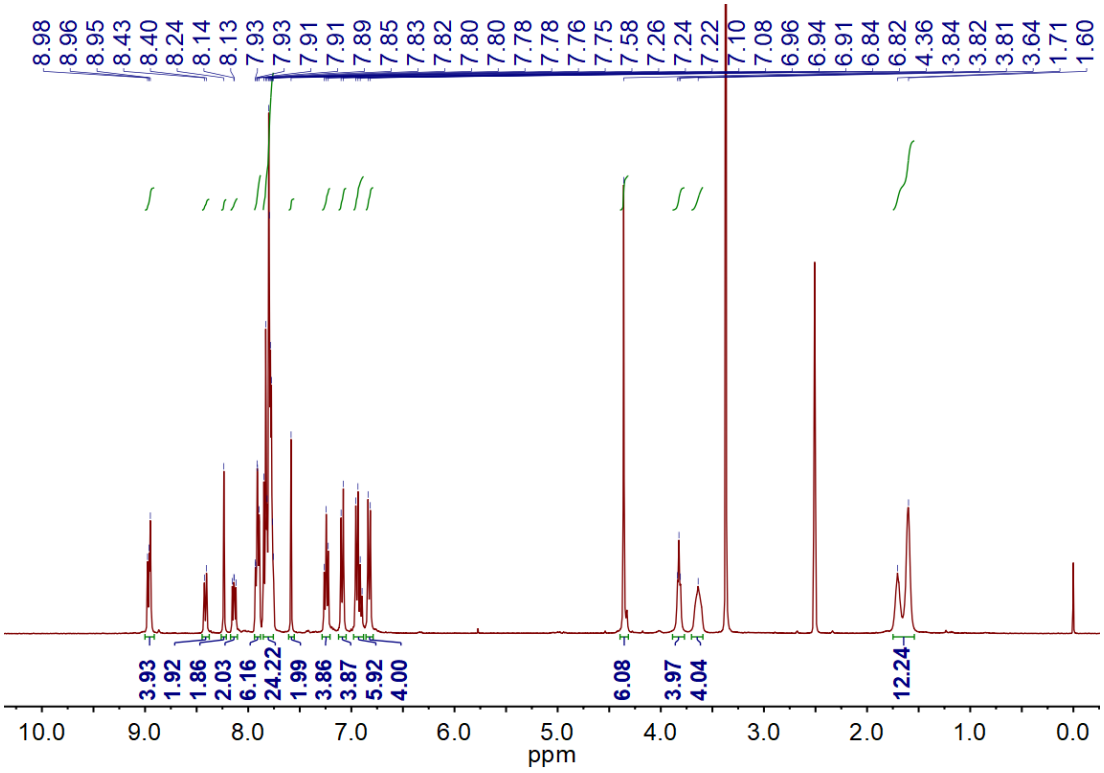


Figure S7. ^{1}H NMR spectrum of OPTM in DMSO-d_6.

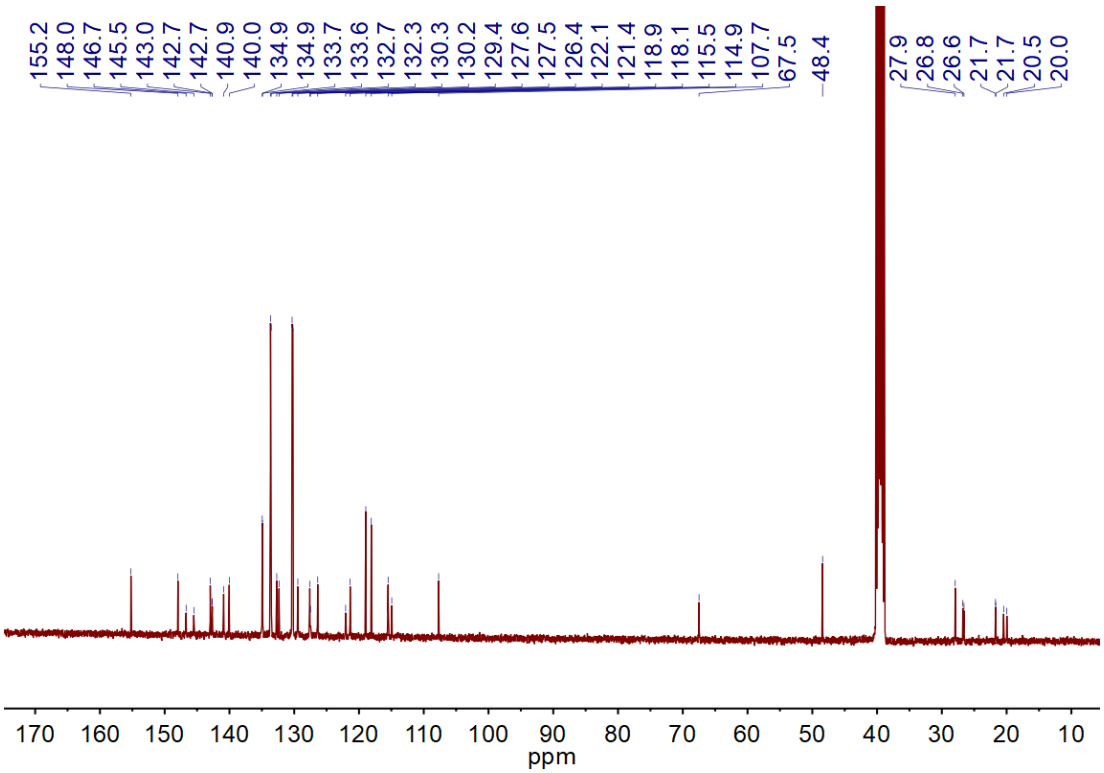


Figure S8. ^{13}C NMR spectrum of OPTM in DMSO-d_6.

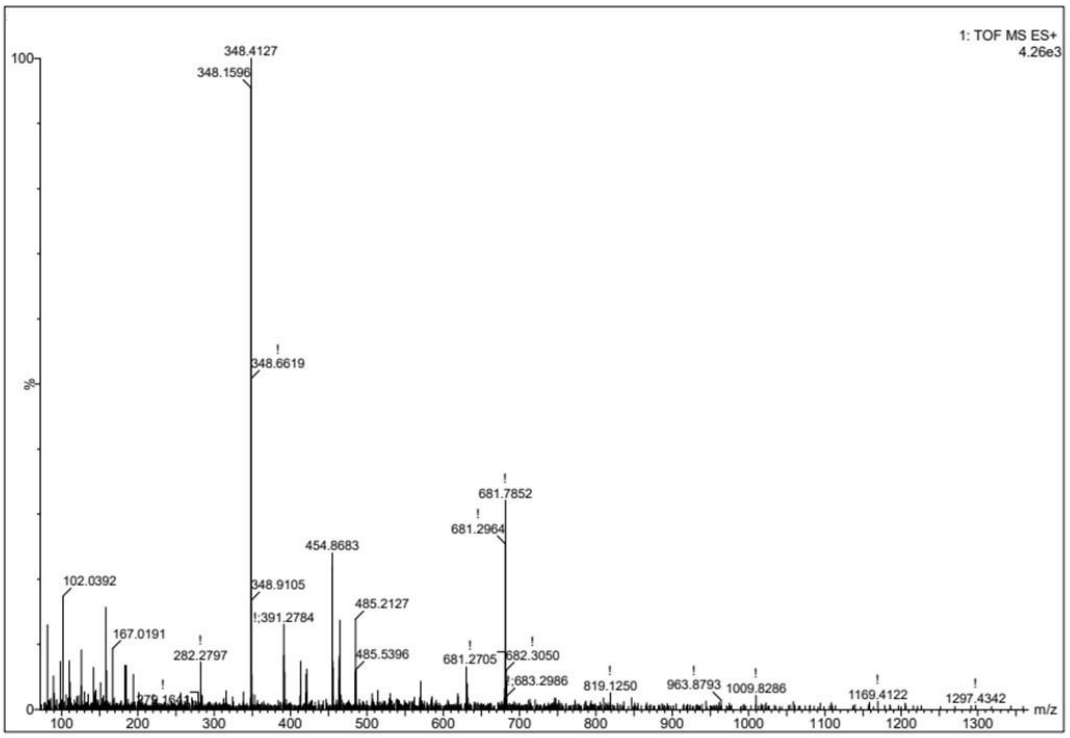


Figure S9. HRMS spectrum of OPTM.

3. Normalized UV/Vis absorption spectra of OPTM

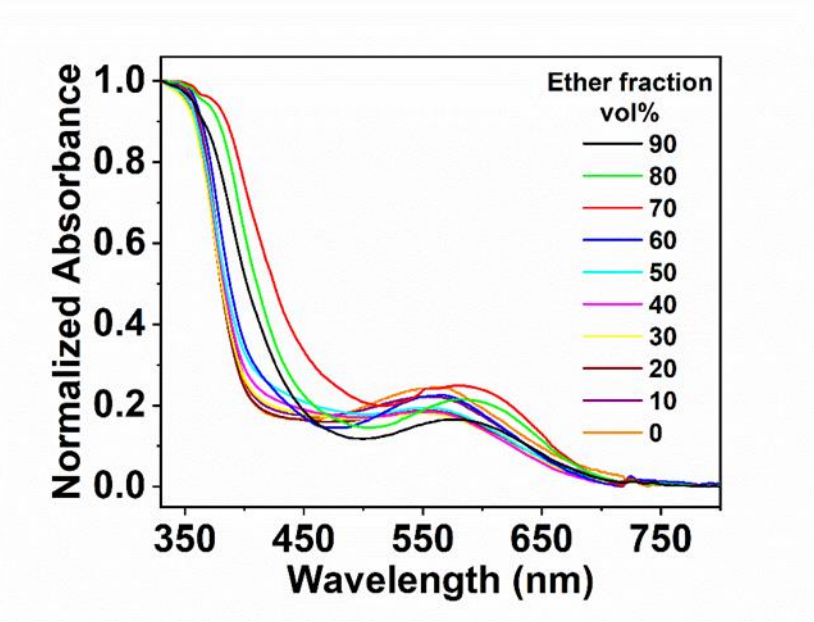


Figure S10. Normalized UV/Vis absorption spectra of OPTM (50 μM) in acetonitrile/ether mixtures with different f_e.

4. DLS of OPTM in acetonitrile/ether mixture

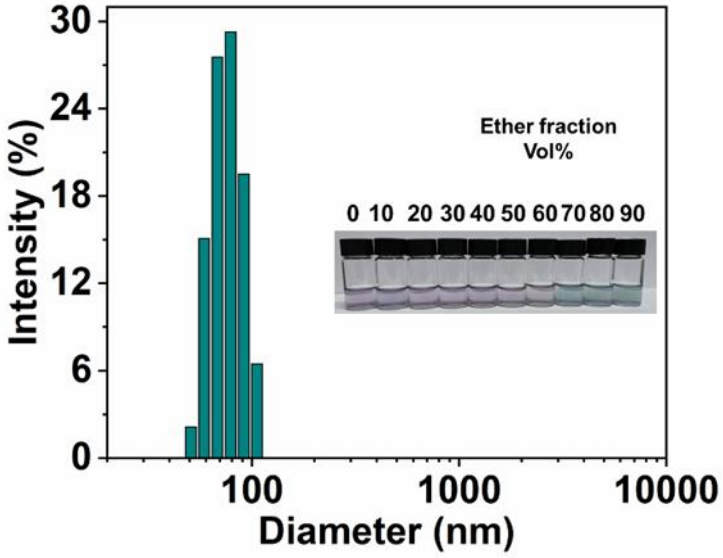


Figure S11. Particle size distribution of OPTM in acetonitrile/ether mixed solution containing 90% ether. Inset: photographs of OPTM (50 μM) in acetonitrile/ether mixtures with different f_e.

5. Average hydrodynamic diameter changes of OPTM NPs

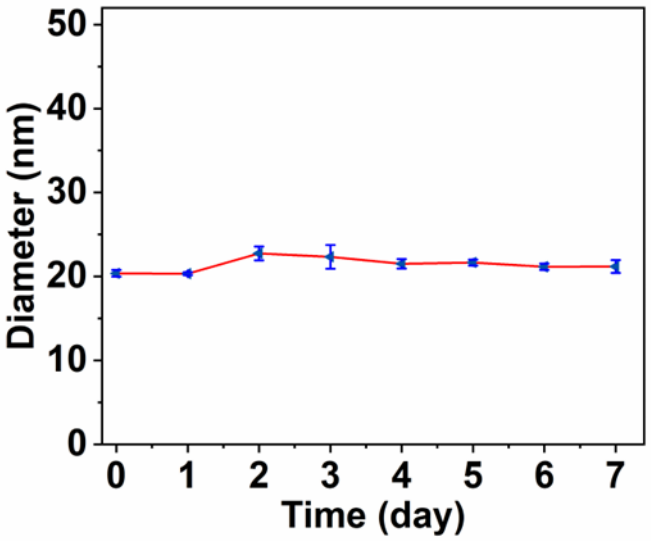


Figure S12. Average hydrodynamic diameter changes of OPTM NPs at different time points at 37ºC.

6. Normalized UV/Vis absorption spectrum of OPTM NPs

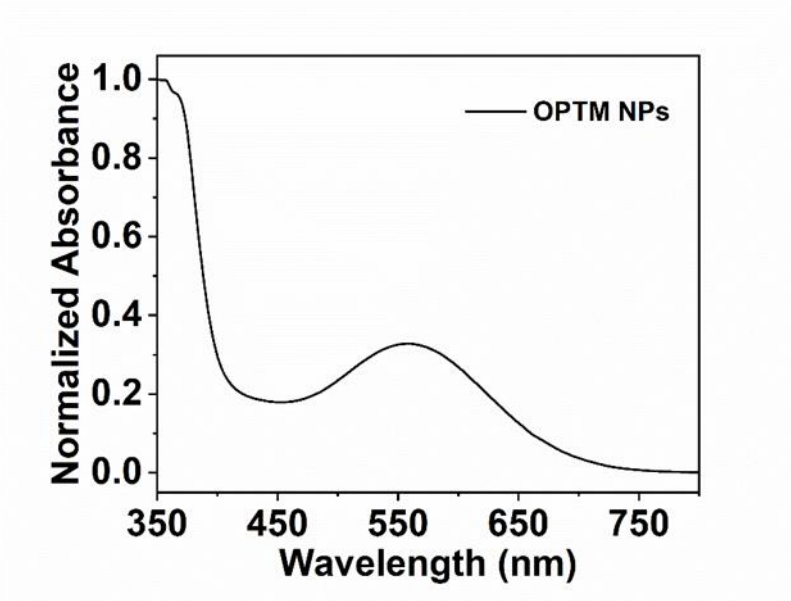


Figure S13. Normalized UV/Vis absorption spectrum of OPTM NPs (0.5 mM).

7. Plot of relative fluorescence intensity (I/I_0) at 525 nm *versus* irradiation time

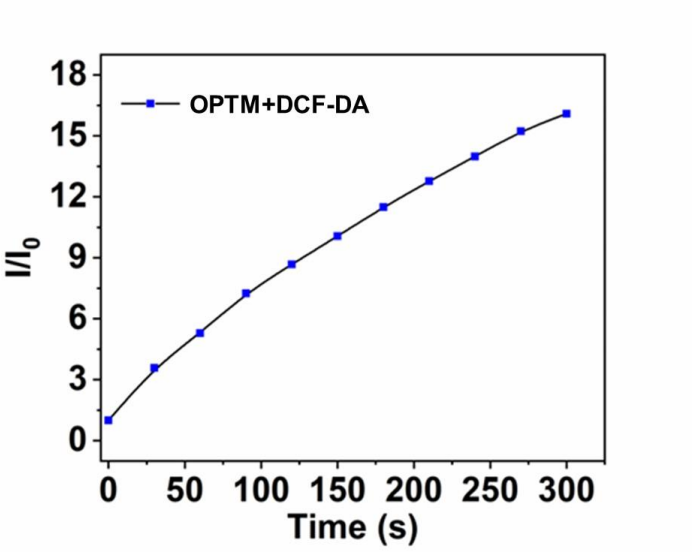


Figure S14. Plot of relative fluorescence intensity (I/I_0) at 525 nm *versus* the irradiation time.

8. Photothermal conversion efficiency of OPTM NPs

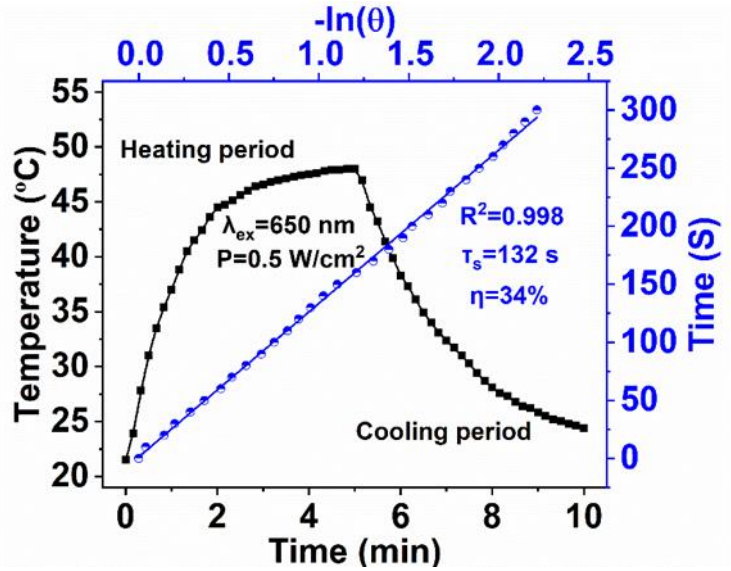


Figure S15. Photothermal conversion efficiency of OPTM NPs. Black line: Temperature-time curve of OPTM NPs under the laser irradiation (0.5 W/cm^2); Blue line: Time constant for heat transfer from the system of OPTM NPs is determined to be τ_s= 132 s by applying the linear time data from the cooling period versus negative natural logarithm of driving force temperature.

9. Colocalization scatterplot of OPTM NPs and Mito-Tracker Green

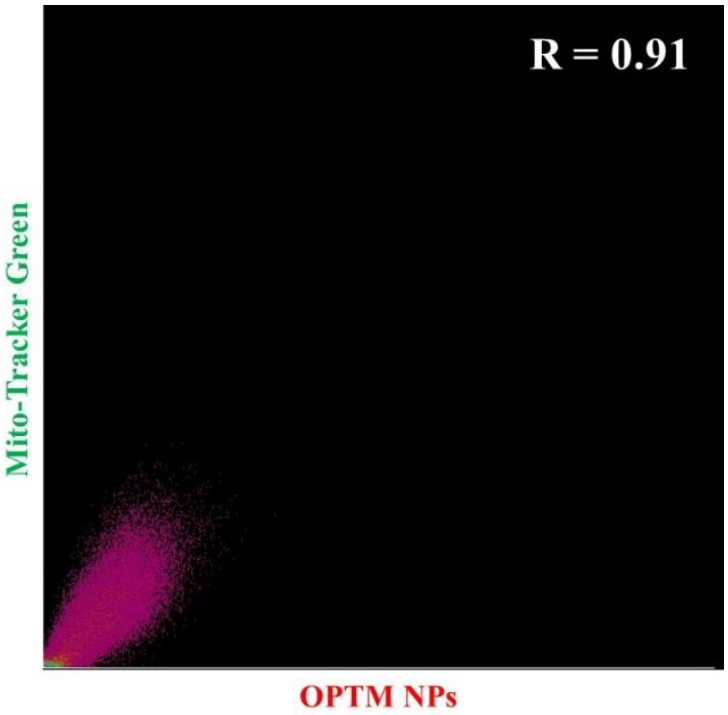


Figure S16. Colocalization scatterplot of OPTM NPs and Mito-Tracker Green.

10. Live/dead staining of OPTM NPs treated 4T1 cells

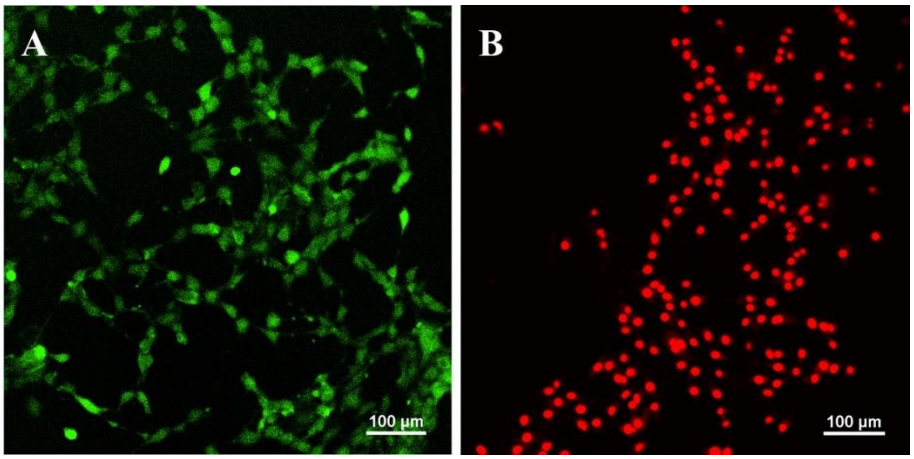


Figure S17. Live/dead staining of OPTM NPs treated 4T1 cells with 650 nm laser irradiation (0.3 W/cm^2) for (A) 0 min and (B) 5 min. The live cells were stained by FDA (green), whereas dead cells were stained by PI (red).

11. Flow cytometric analysis of 4T1 cells treated with OPTM NPs

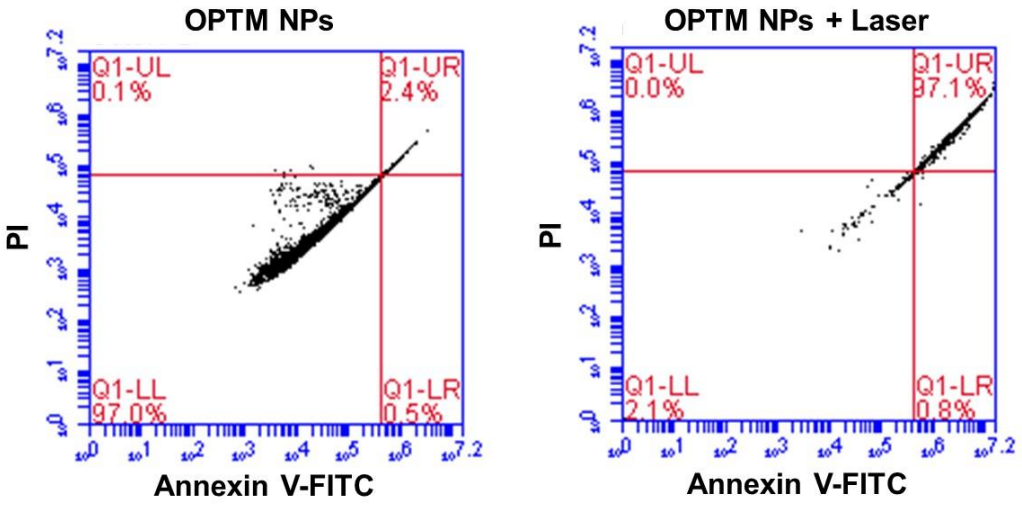


Figure S18. Flow cytometric analysis of 4T1 cells treated with OPTM NPs with or without laser irradiation (650 nm, 0.3 W/cm^2).

References

(1) Lin, H.; Gao, S.; Dai, C.; Chen, Y.; Shi, J. A Two-Dimensional Biodegradable Niobium Carbide (MXene) for Photothermal Tumor Eradication in NIR-I and NIR-II Biowindows. *J. Am. Chem. Soc*. **2017**, *139*, 16235-16247.