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A photo-triggered antifungal nanoplatform with efflux pump and heat shock protein reversal activity for enhanced chemo-photothermal synergistic therapy

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Materials and Methods

Materials

Ovolecithin and 1-hexadecanol (99%) were separately procured from Adamas-Beta and Sigma-Aldrich. FLU and 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) were provided by Macklin (Shanghai, China). DSPE-PEG_{2000} was bought from Xi'an ruixi Biological Technology Co., Ltd (Xi'an, China). Fetal bovine serum (FBS) was procured from Gibco. BDSF was synthesized according to our previously described method.[1] The synthetic route of 4-(2-((3-(3,4,5-trimethoxyphenyl))-5-(4-methoxyphenyl)-1H-pyrrol-2-yl)imino)-5-(4-methoxyphenyl)-2H-pyrrol-3-yl)-N, N-diethylaniline (TMOB) was illustrated in **Fig. S1**.

Synthesis of TMOB

*Synthesis of compound **1***: Potassium hydroxide (0.06 g, 1.0 mmol), 4-methoxyacetophenone (1.50 g, 10 mmol) and 3,4,5-trimethoxybenzaldehyde (1.96 g, 10 mmol) were dissolved in 50 mL of ethanol/water mixed solvent (4:1) and stirred at room temperature for 24 h. The product was collected by filtering and washing with cold ethanol to give the yellow solid (1.835 g, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 8.038 (d, *J* = 9.2 Hz, 2H, -CH=C), 7.712 (d, *J* = 15.2 Hz, 1H, Ar-CH=), 7.419 (d, *J* = 16.0 Hz, 1H, CH-C=O), 6.988 (d, *J* = 8.8 Hz, 2H, Ar-H), 6.861 (s, 2H, Ar-H), 3.910 (d, *J* = 10.0 Hz, 12H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 195.473, 163.955, 153.596, 135.054, 129.515, 113.972, 104.523, 77.471, 77.155, 56.255, 55.622, 41.344, 39.859.

*Synthesis of compound **2***: Compound **1** (3.28 g, 10.0 mmol) in methanol (60 mL) was mixed with diethylamine (2 mL, 50.0 mmol) and nitromethane (2.5 mL, 50.0 mmol), then heated at

75 ℃. After reflux for 48 h, the reaction was halted, the sample was cooled and acidified with 2 M hydrochloric acid. After extracting with dichloromethane (CH_2Cl_2, DCM), the organic layer was dried with anhydrous sodium sulfate (Na_2SO_4) and evaporated to give the white oily crude product (2.61 g, 67%). The resulting product was used directly for further experiment. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.907 (d, *J* = 9.2 Hz, 2H, Ar-H), 6.928 (d, *J* = 9.2 Hz, 2H, Ar-H), 6.459 (s, 2H, Ar-H), 4.752 (m, 2H, CH-C=O), 4.151 (m, 1H, Ar-CH), 3.868 (s, 3H, OCH_3), 3.838 (s, 6H, OCH_3), 3.806 (s, 3H, OCH_3), 3.361 (m, 2H, -CH_2-NO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 195.473, 163.955, 153.596, 135.054, 129.515, 113.972, 104.523, 77.471, 77.155, 56.255, 55.622, 41.344, 39.859.

*Synthesis of compound **3***: Compound **2** (0.25 g, 0.64 mmol), ammonium acetate (1.97 g, 20 mmol) and butanol (10 mL) were added into a 100 mL round-bottomed flask and heated at 130 ℃ under reflux for 18 h. The reaction was cooled to room temperature and filtered. The precipitate was washed with ethanol to obtain a dark green solid (0.130 g, 59%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.907 (d, *J* = 9.2 Hz, 4H, Ar-H), 7.103 (m, 10H, Ar-H), 3.917 (s, 12H, OCH_3), 3.718 (s, 12H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 161.435, 154.219, 153.060, 149.255, 142.471, 138.063, 129.726, 128.250, 125.021, 114.355, 106.315, 77.433, 76.791, 61.027, 55.986, 55.622.

Synthesis of TMOB: 80 mL of anhydrous CH_2Cl_2 supplemented with diisopropylethylamine (0.71 g, 5.5 mmol) and compound **3** (0.34 g, 0.5 mmol) was chareged with nitrogen gas. Under stirring condition, the boron trifluoride diethyl etherate (1.12 g, 7.9 mmol) was injected slowly. After stirring for 24 h at room temperature, the solution was washed with methanol. The organic layer was harvested, dehydrated with anhydrous Na_2SO_4, and

evaporated. Then a red solid was harvested after purification by using column chromatography (0.31 g, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 8.088 (d, *J* = 12.4 Hz, 2H, Ar-H), 7.188 (s, 2H, Ar-H), 7.019 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.960 (s, 1H, pyrrole-H), 3.907 (d, *J* = 10.0 Hz, 6H, OCH_3), 3.769 (s, 6H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 162.019, 157.966, 153.280, 145.374, 143.506, 139.443, 131.709, 128.298, 124.158, 118.552, 114.336, 106.660, 77.442, 77.126, 76.810, 61.104, 56.073, 55.527. MALDI-TOF (m/z): calcd for [$C_{40}H_{38}BF_2N_3O_8$], 737.272; found, 737.425.

Preparation of TMOB/FLU@PCM nanoparticles

The TMOB/FLU@PCM NPs were fabricated through a resolidification method.[2] In detail, 2 mg TMOB, 4 mg FLU, and a certain amount of PCM were dissolved in methanol and tetrahydrofuran mixed solution (1:1). The above solution was dropped to 10 mL of Mili-Q water containing ovolecithin (20 mg) and DSPE-PEG_{2000} (30 mg) under a 50 ℃ ultrasonic water bath. Then the obtaining solution was cooled instantly using ice bath to solidify the nanoparticles. After dialyzed and filtrated with a 0.45 μm filter, TMOB/FLU@PCM NPs were obtained and stored at 4 ℃ for further application. A similar procedure was carried out to fabricate PCM NPs, TMOB@PCM NPs and FLU@PCM NPs.

Photothermal effect of TMOB/FLU@PCM NPs

TMOB/FLU@PCM NPs was diluted with PBS buffer. Then 100 μL of TMOB/FLU@PCM NPs solution was added to 200 μL PCR reaction tube and irradiated with 808 nm laser (0.328 W cm^{-2}) for 7 min. To investigate the photothermal property of TMOB/FLU@PCM NPs, 100 μL of TMOB/FLU@PCM NPs solution (200 μg mL^{-1}) was exposed to different power densities (0.084, 0.145, 0.198, 0.256, 0.325 W cm^{-2}). The temperature of

TMOB/FLU@PCM NPs solution was monitored using an infrared imaging device. To probe the size of TMOB/FLU@PCM NPs after laser irradiation, the TMOB/FLU@PCM NPs solution was characterized by dynamic light scattering (DLS).

Drug releasing

UV-vis-NIR spectrometer was implemented to measure the loading efficiency of TMOB and FLU. The release behavior of FLU was monitored using UV-vis-NIR spectrophotometer when TMOB/FLU@PCM NPs were treated with or without 808 nm laser irradiation (0.325 W cm^{-1}), while the release profile of BDSF from TMOB/FLU@PCM NPs was measured with high-performance liquid chromatography (HPLC).

***C. albicans* culture**

ARCA strain Nos. 108 was used in this study.[3] *ARCA* cells were inoculated into 5 mL of YPD (1% Yeast extract, 2% Peptone, and 2% Dextrose) medium. After overnight culture at 35 ℃, 220 rpm, the fungal cells were collected and re-dispersed in PBS buffer for the next use. The concentration of fungal cells was measured with a hematocytomete under microscope.

In vitro antifungal assay

The fresh fungal cells were diluted to 10^5 CFU mL^{-1} with (10% YPD) and treated with a series (0, 20, 40 μg mL^{-1}) of concentration's nanomaterials for 2 h at 35 ℃. After the samples treated with 808 nm laser (0.328 W cm^{-2}) for 10 min, the fungal cells were cultured in a microbial incubator for another 12 h. Then the cell density was measured using XTT assay.[4] For the plate colony counting, the sample was diluted with PBS and spread on YPD plate after treated with nanomaterials and 808 nm laser. For exploration the change of fungal

cell morphology, about 1×10^6 CFU mL^{-1} *ARCA* cells were treated with 808 nm laser and nanomaterials (PCM NPs, TMOB@PCM NPs, FLU@PCM NPs, TMOB NPs + FLU, and TMOB/FLU@PCM NPs) simultaneously. Then the fungal cells were collected, washed with PBS for twice and fixed by 2.5% glutaraldehyde for 2 h. Next, the cells were dehydrated with different concentrations of ethanol solution (20, 40, 60, 80, 90 and 100%). Finally, the cells were dripped onto silicon plate and characterized using a scanning electron microscope (SEM).

Hyphal formation assay

The effect of nanomaterials on *ARCA* filament formation was performed in YPD medium containing 10% FBS. *ARCA* cells were diluted to 5.0×10^5 cell mL^{-1} using YPD medium supplemented with 10% FBS.[5] Then, the nanoparticles were added to 96 well plate and irradiated with 808 nm laser (0.328 W cm^{-2}) before the fungal cells were added to the each well. And the plate was cultured at 37 ℃ for 4 h and photographed using a microscope.

Biofilm development

3 mL of YPD medium containing 5% FBS and *ARCA* cells (1.0×10^6 cell mL^{-1}) were added into confocal dishes and cultured at 37 ℃ without shaking. One hour after adhesion, the old medium was removed and rinsed with PBS to eliminate the unbound *ARCA* cells. Then the fresh medium was reinfused and incubated at 37 ℃ for biofilm development. To harvest the mature biofilm, the biofilm was rinsed gently to remove the loosely unbound fungal cells after 2 days' cultivation.[6]

Biofilm inhibition and eradication

To explore the suppression effect of nanoparticles on biofilm development, the PCM NPs, TMOB@PCM NPs, FLU@PCM NPs, TMOB NPs + FLU, and TMOB/FLU@PCM NPs were treated with or without laser (0.328 W cm^{-2}) for 10 min. Then the result suspensions were added to YPD medium containing 5% FBS for culture *ARCA* cells at 37 ℃. After 24 h incubation, the resulting biofilm was stained by crystal violet. To investigate the disruption effect on biofilm, *ARCA* biofilm was constructed in advance. Then the medium containing PCM NPs, TMOB@PCM NPs, FLU@PCM NPs, TMOB NPs + FLU, and TMOB/FLU@PCM NPs was reinfused and irradiated with or without 808 nm laser (0.328 W cm^{-2}, 15 min). After receiving different treatments, the surviving biofilm was rinsed gently with PBS buffer, stained by Calcein-AM and crystal violet, respectively.[4]

Real-time RT-PCR

The gene sequence was retrieved from *Candida* Genome Database (http://www.candidagenome.org/). The primers employed in this work were designed by using Primer Premier as displayed in Table S1. For RT-PCR analysis, *ARCA* cells were treated with PBS, TMOB NPs + FLU and TMOB/FLU@PCM NPs (40 μg mL^{-1}) under 808 nm laser irradiation and cultured for another 10 h. Then the cells were collected and total RNA was harvested via an RNA fast extracting kit (Tiangen Biotech Co.). Then the cDNA was prepared by using revertAid™ first strand cDNA synthesis kit for quantitative analysis of target gene expression as our previous described.[3]

***In vivo* antifungal experiment**

Twenty-five BALB/c female mice were procured from Qinglong mountain experimental animal center (Nanjing, China), and all mice were raised and used under the guidelines

approved by the School of Pharmaceutical Science (ratification No. LL-20190305-05), Nanjing Tech University. *ARCA*-infected mice model was constructed by injecting fungal cell (1×10^7 cells, 100 μL) subcutaneously into the right back of mice. After infection for 4 days, a subcutaneous abscess could be seen in each mouse. The infected mice were apportioned equally to each group. Then the NPs (200 μg mL^{-1}, 50 μL) was subcutaneously injected into the abscessed site and treated with 808 nm laser (0.325 W cm^{-1}, 10 min). Afterwards, the mice were weighted and photographed. After 12 days of therapy, the mice were euthanatized, and the abscess tissues were harvested, homogenized, and spread on YPD plates to measure the population of *ARCA* in the abscesses. For pathological analysis, the abscess tissues were fixed by 4% paraformaldehyde, paraffined, sectioned for Periodic Acid-Schiff (PAS) and Masson's trichome staining.

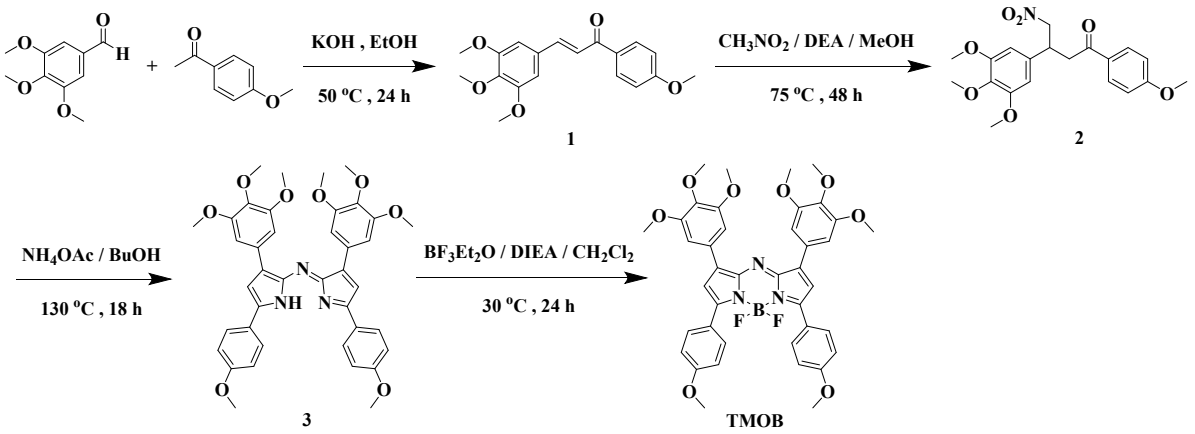

Fig. S1. Synthetic procedure for TMOB.

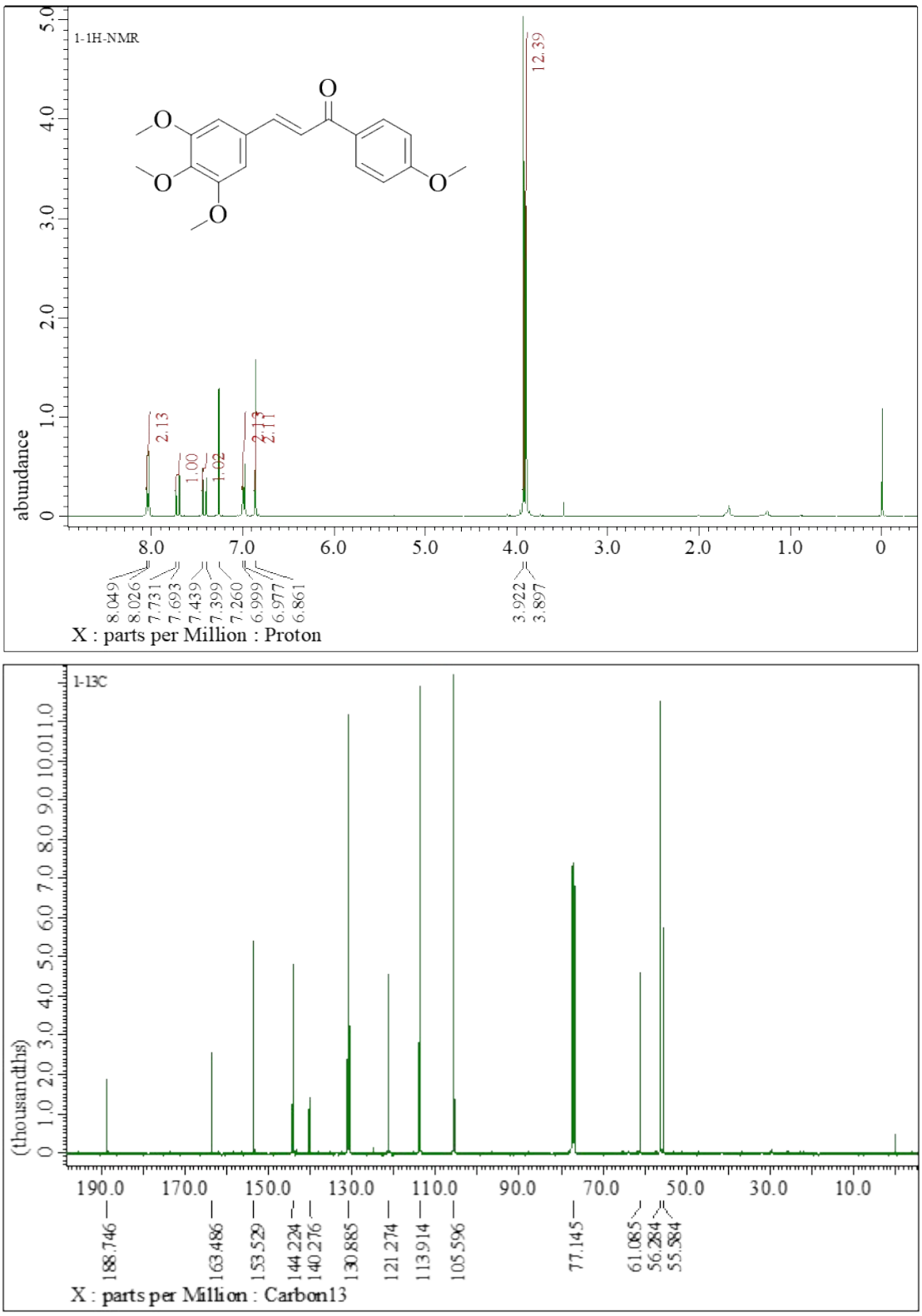

Fig. S2. ^{1}H NMR (up) and ^{13}C NMR (down) spectra of **1**.

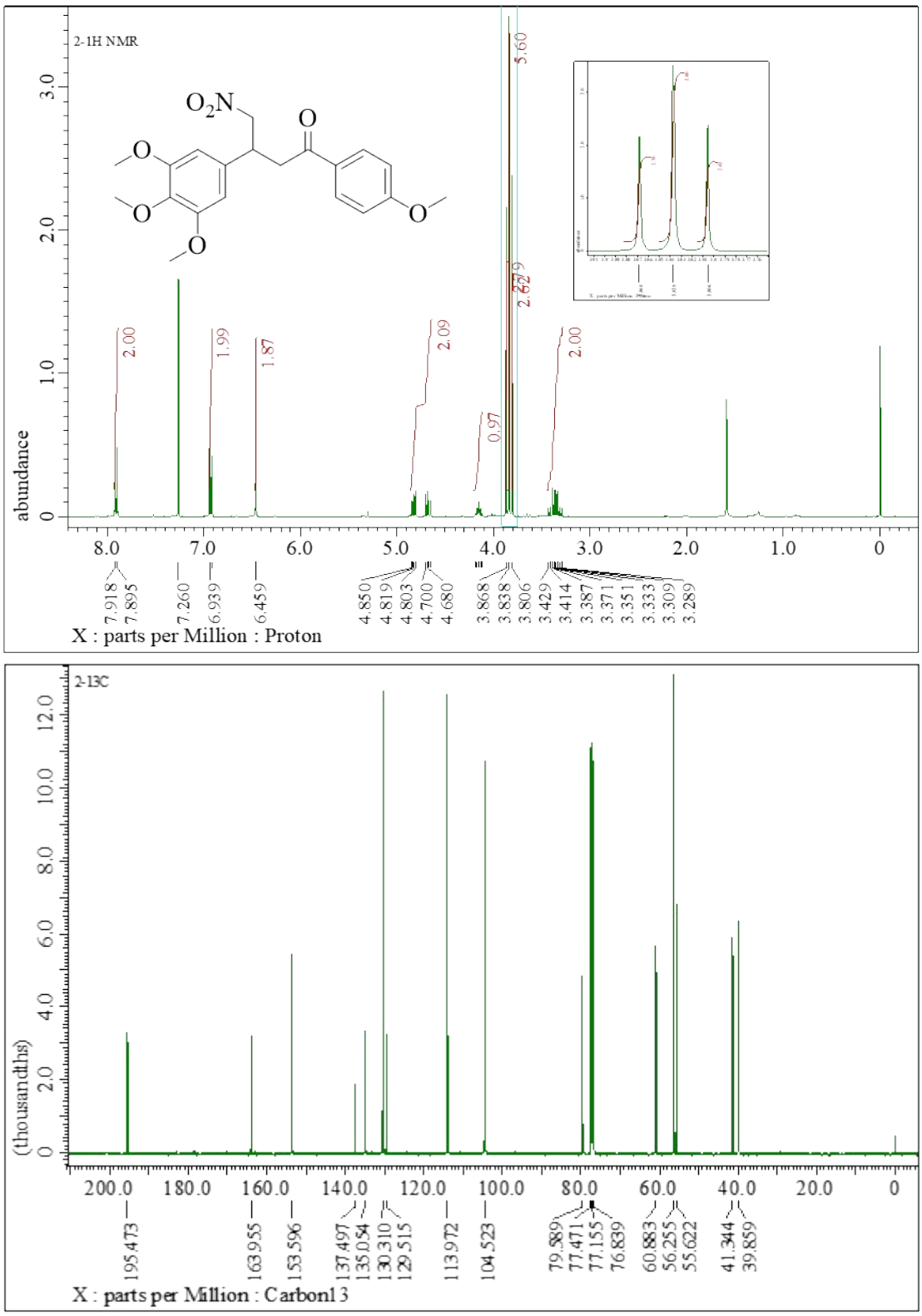

Fig. S3. ^{1}H NMR (up) and ^{13}C NMR (down) spectra of **2**.

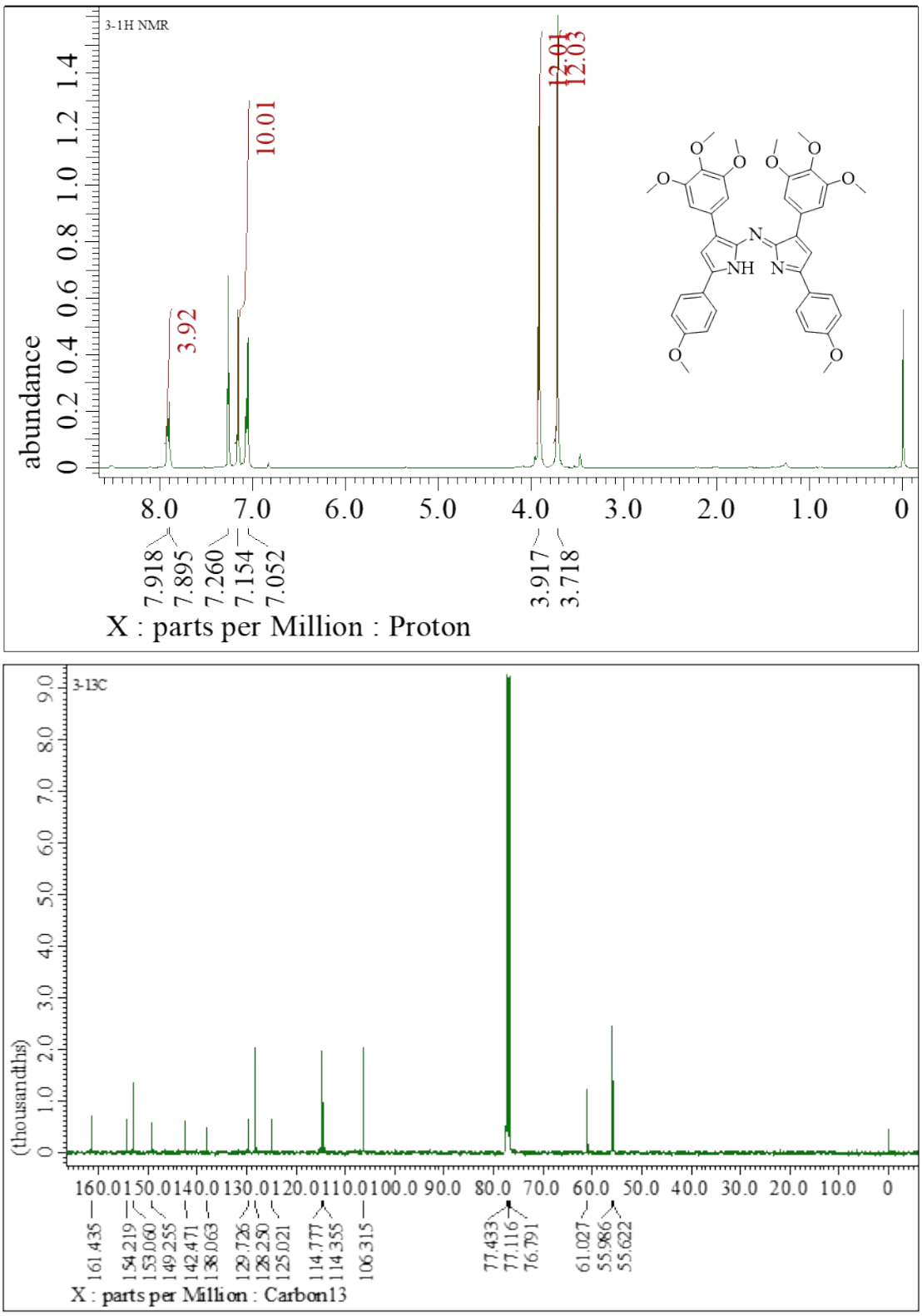

Fig. S4. ^{1}H NMR (up) and ^{13}C NMR (down) spectra of **3**.

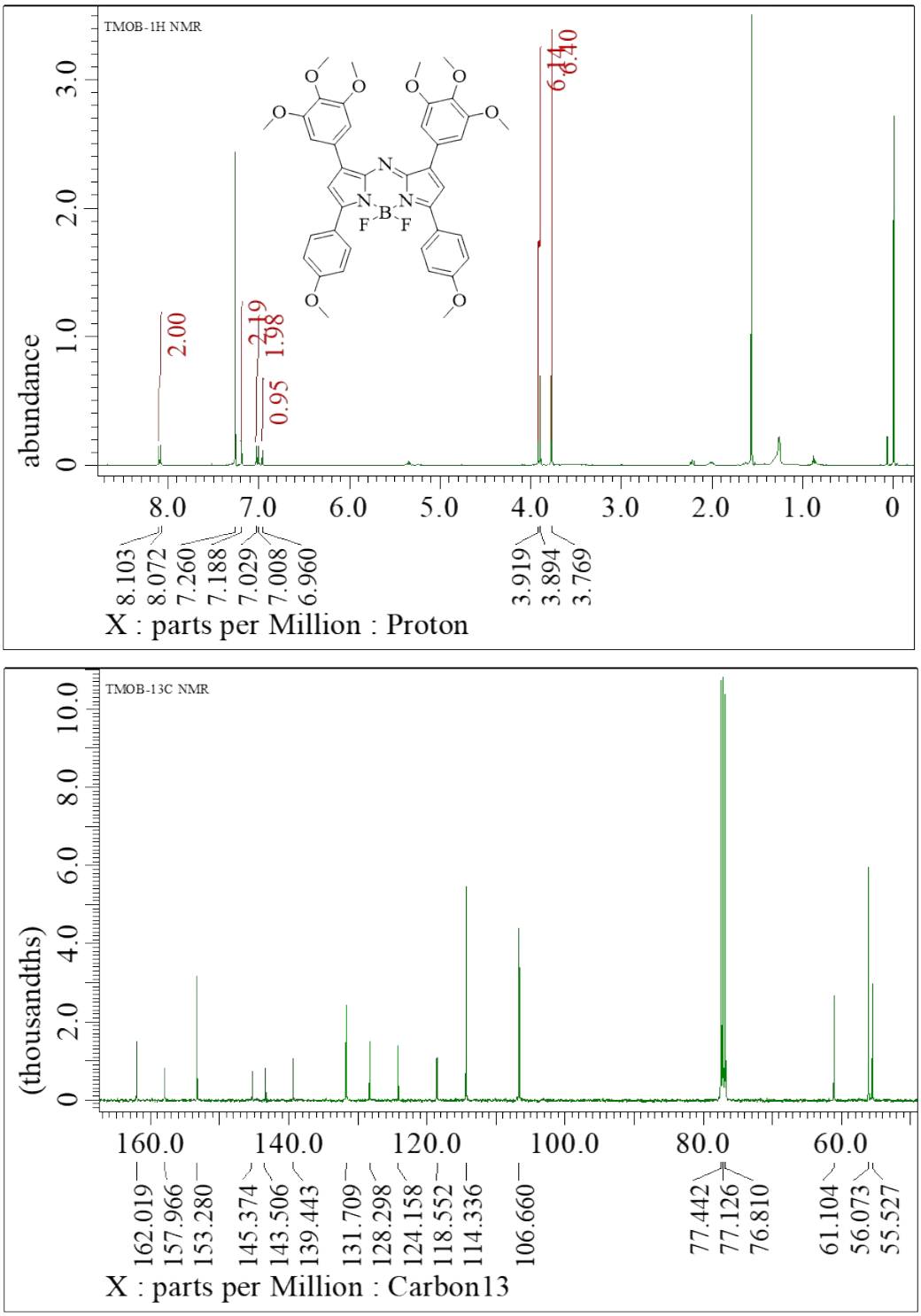

Fig. S5. ^{1}H NMR (up) and ^{13}C NMR (down) spectra of TMOB.

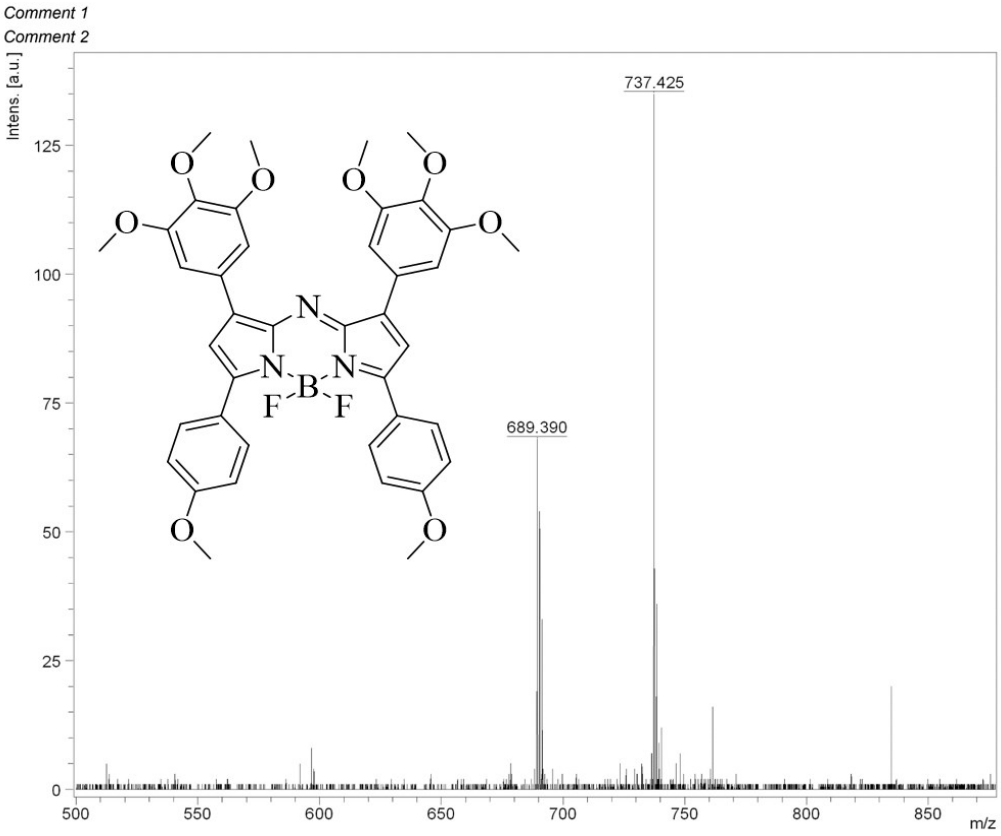

Fig. S6. MALDI-TOF spectrum of TMOB.

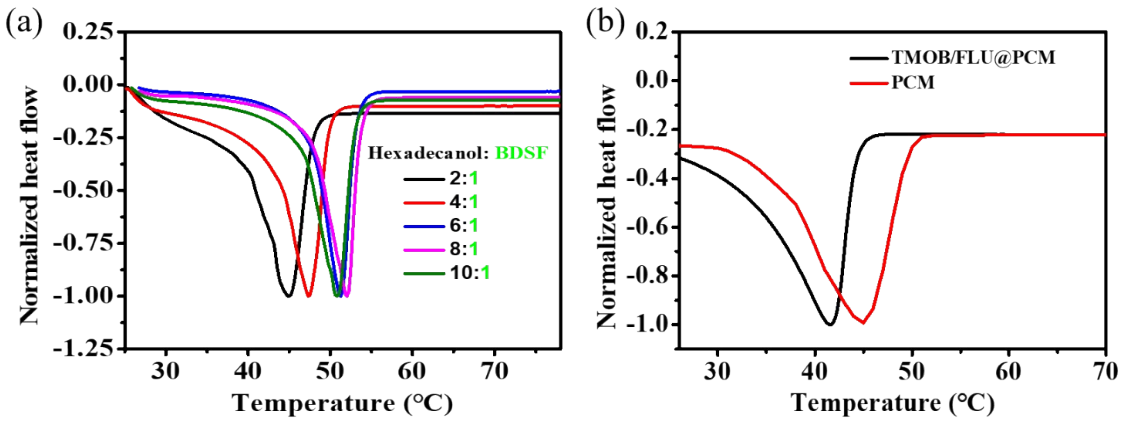

Fig. S7. (a) DSC curves of PCM with different hexadecanol/BDSF mass ratios. (b) DSC curves of PCM (hexadecanol/BDSF = 2/1) before and after loading TMOB and FLU.

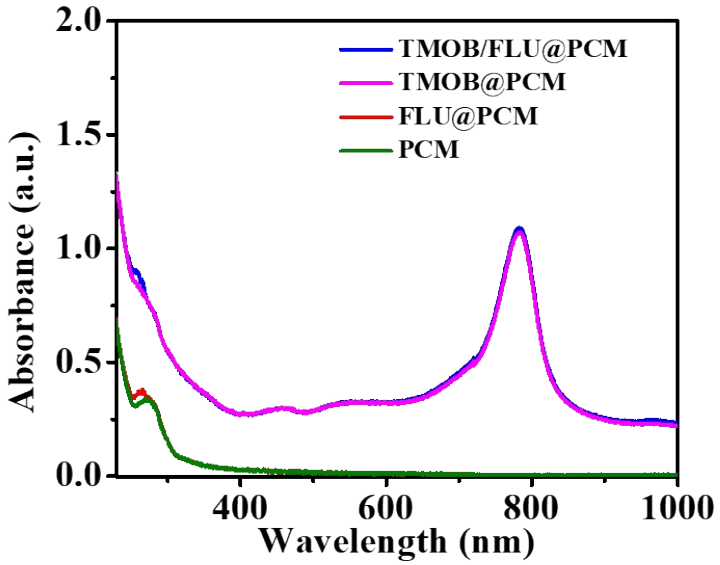

Fig. S8. Absorption spectra of TMOB/FLU PCM NPs, TMOB PCM NPs, FLU PCM NPs and PCM NPs (10 μg mL^{-1}).

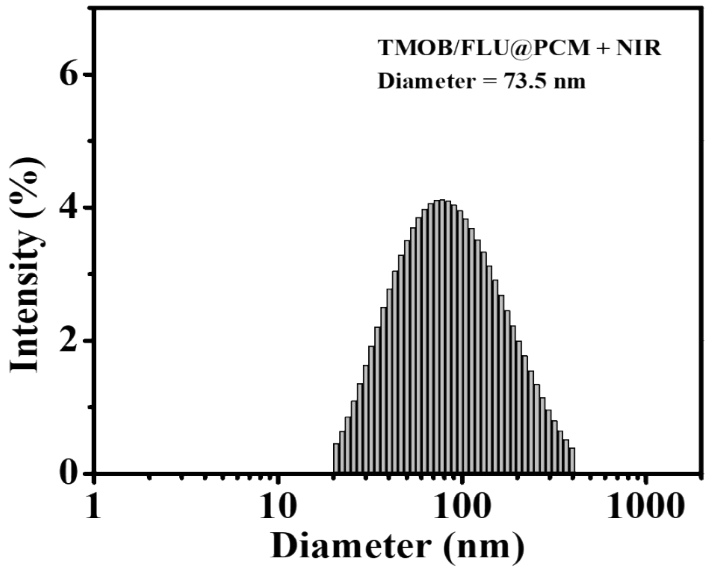

Fig. S9. Hydrodynamic diameter of TMOB/FLU@PCM NPs after irradiation with 808 nm laser.

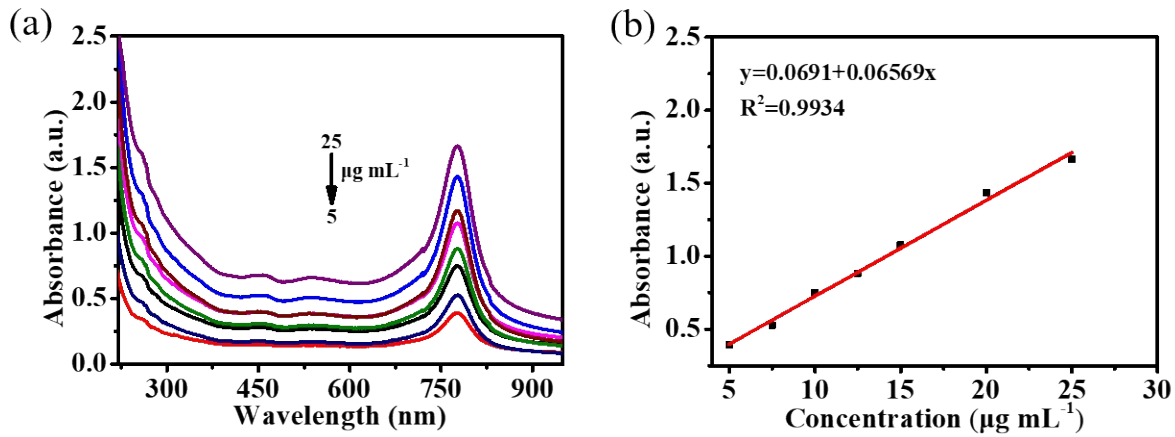

Fig. S10. (a) Absorption spectra of TMOB/FLU@PCM NPs with different concentrations (5, 7.5, 10, 12.5, 15, 20 and 25 μg mL^{-1}). (b) Linear absorbance value at 778 nm versus the concentration of TMOB/FLU@PCM NPs.

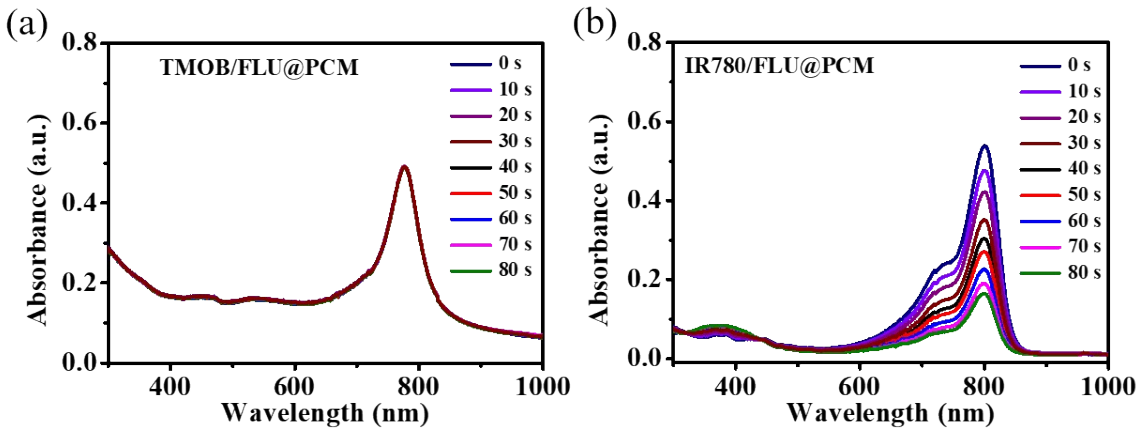

Fig. S11. Absorption spectra of (a) TMOB/FLU@PCM NPs and (b) IR780/FLU@PCM NPs under 808 nm laser irradiation.

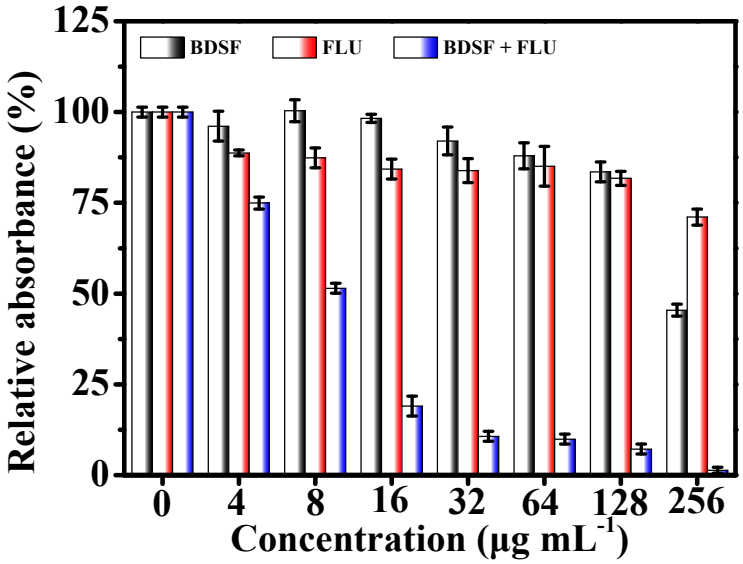

Fig. S12. The synergistic antifungal performance of BDSF and FLU, the concentration of BDSF and FLU is the same.

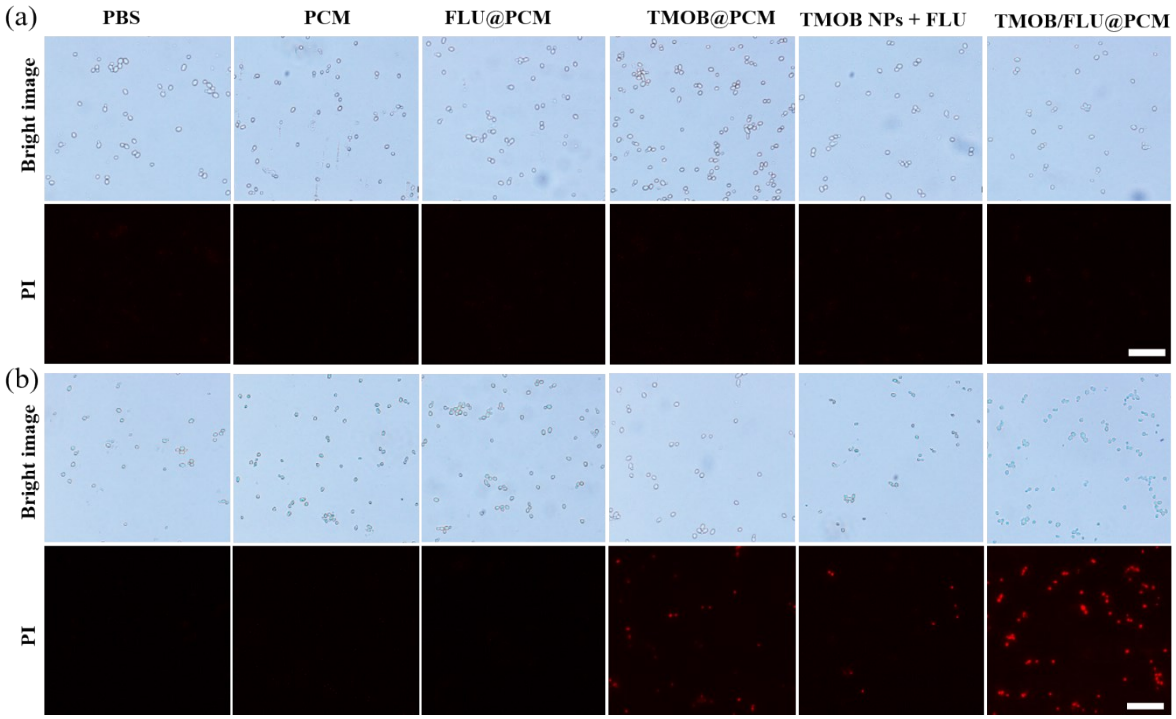

Fig. S13. Dead cell analysis of *ARCA* cells after various treatments. (a, b) Without and with laser irradiation. Scar bar: 50 μm.

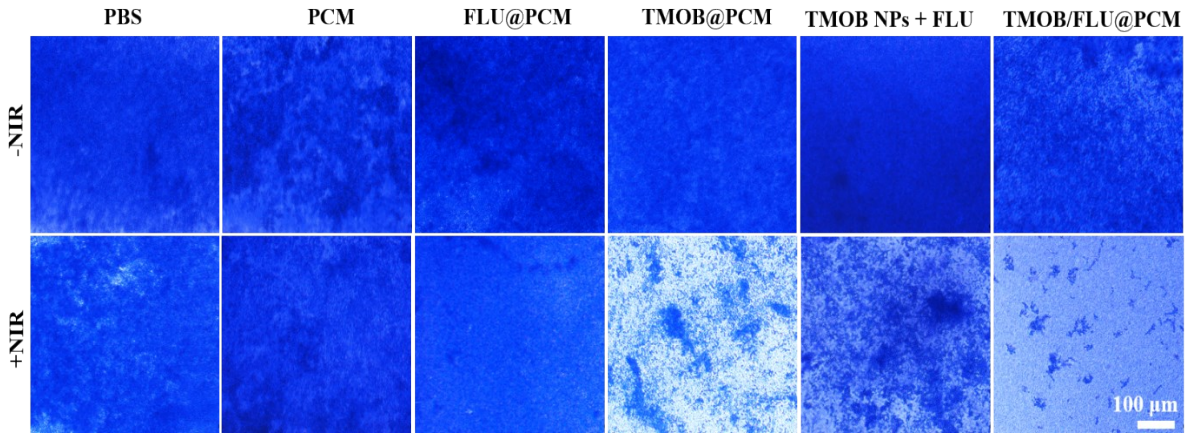

Fig. S14. Inhibition biofilm formation assay under YPD + 10% FBS medium at 37 ℃.

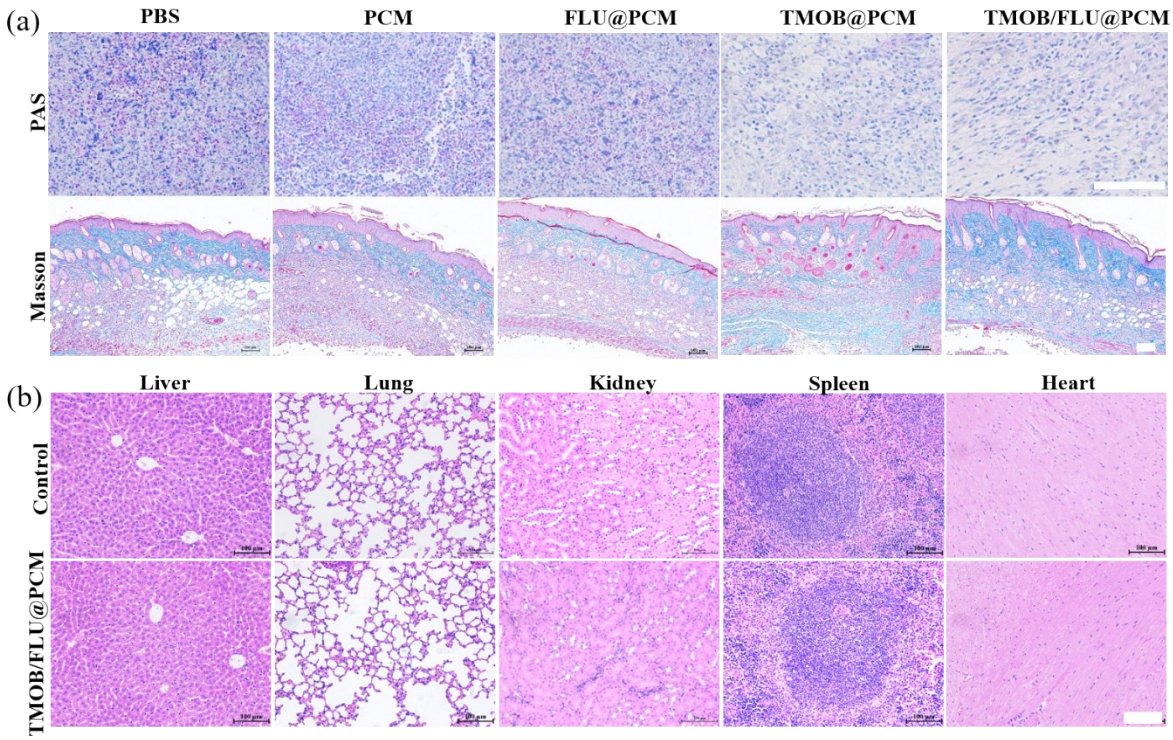

Fig. S15. (a) PAS, Masson's trichrome stained images of the abscessed tissues in different treatment groups. (b) H&E stained images of the main organs of the mice after different treatments. Scale bar: 100 μm.

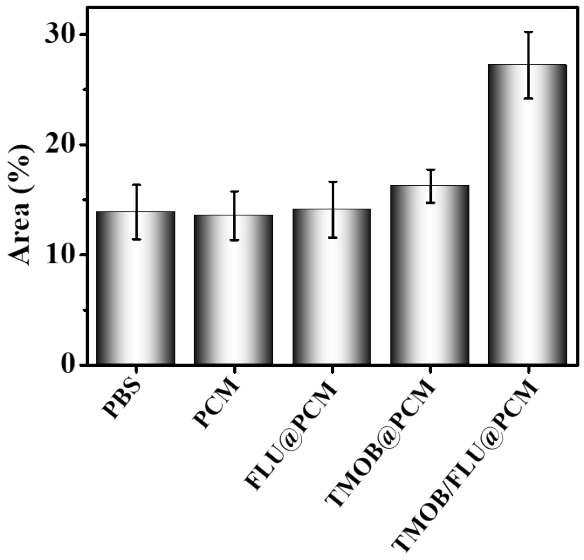

Fig. S16. The quantification analysis of collagen fibers in Masson's trichrome staining.

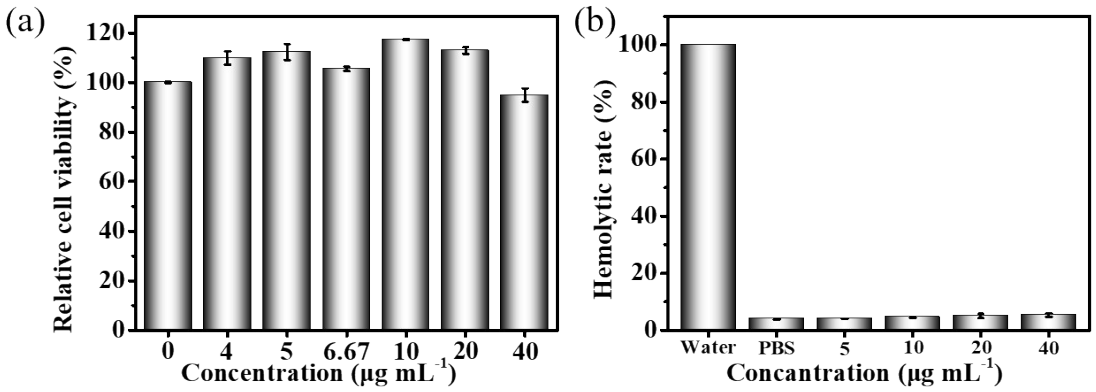

Fig. S17. (a) The cytotoxicity of TMOB/FLU@PCM NPs towards HaCaT cells. (b) The hemolytic rate of TMOB/FLU@PCM NPs.

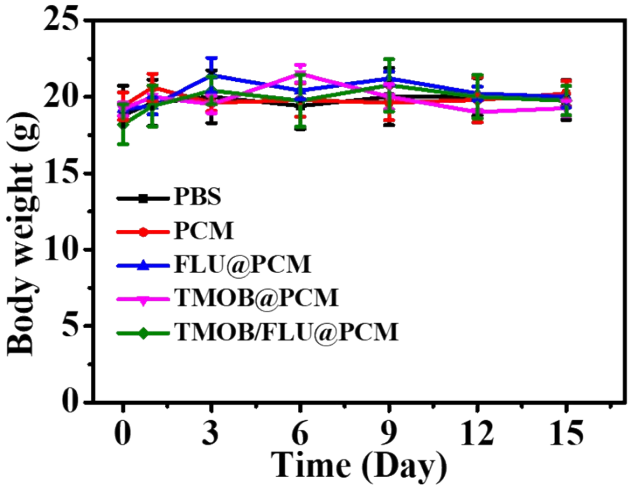

Fig. S18. Body-weight changes of mice after various treatments and laser irradiation.

Table S1. Primers used in this study

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	PCR Products (bp)
ACT1	CAACTGGGACGATATGGAAAA	TGGAAACGTAGAAAGCTGGAA	176
MDR1	TTTTGCGTCAAGAACAGGTTT	GCACCAGAAGCAGTAGTAGCAG	160
CDR2	TGGGTGGATGCACTGGAC	GGCTAAATCTTGGATGTTGTGA	84
CDR4	ACGTTTGAACCCGAACTCAC	AGAAGCCGAACCATAAGCAC	145
CDR1	TGGTGTCGCTGGTGAAGG	CGACCAGGCAGTTTGAGAAT	136
HSP12	GGCAAGGAACAAGTCACCAG	GTCTTTGCTTCGCCCTCTG	167
HSP21	TGCCACTGAAGTTCCACCA	TTGCCAATGCTCTGTGAGG	119
HSP60	CGTTGCCAAAGCCATCAC	ACCGTCACCAGCACTTTCAT	100
HSP90	AGTGCTGGTGCTGACGTTTC	CGTATTGTTCGTCGTCATTGTG	115

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