All-in-One Mitochondria-targeted NIR-II Fluorophores for Cancer Therapy and Imaging

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Author contributions

X. Hong conceived and designed the experiments. Y. Zheng, Q. Li, A. Li, Z. Luo, J. Wu, Y. Chen, W. Zhou, T. Rouzi, T. Tian, H. Zhou, X. Zeng, Y. Li, X. Cheng performed the experiments. Q. Li, Y. Zheng, F. Zhou, X. Hong, Z. Deng analyzed the data, Q. Li, Y. Zheng, X. Hong wrote the manuscript. All authors discussed the results and commented on the manuscript.

Materials and General Procedure

The synthesis reagents were purchased from commercial suppliers (such as Aldrich, Adamas, Energy Chemical, Sinopharm Group Co., Ltd.) and used without further purification unless otherwise noted. And N_3-PEG_8-NHS was purchased from Shanghai Pengsheng Biotechnology Co., Ltd. Dimethylformamide (DMF) were distilled from calcium hydride. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ / CD_3CN at room temperature using a Bruker AV400 magnetic resonance spectrometer. MALDI-TOF-MS characteristics were performed on an AB SCIEX 5800 MALDI

TOF mass spectrometer. Analytical TLC were performed on silica gel plates, and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals. UV-vis-NIR spectra were tested with a SHIMADZU UV-2600 or PerkinElmer Lambda 25 spectrophotometer. The purification of **H4-PEG-Glu** were conducted under Dionex Ultimate 3000 HPLC system. Cell confocal images were captured by a Leica-LCS-SP8-STED confocal laser scanning fluorescence microscope (Leica, Germany). NIR fluorescence spectrum was performed on an Applied Nano Fluorescence spectrometer at room temperature with an excitation laser source of and 808 nm. The NIR-II *in vivo* imaging system was purchased from Suzhou NIR-Optics Technologies CO., Ltd.

Ethics Statement

AML patient derived PDX mouse models construction and all animal experiments were approved by the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals and Institutional Animal Care and Use Committee (IACUC) of Wuhan University. The study has been approved by the Institutional Ethical Committee of Zhongnan Hospital of Wuhan University, China (No. 2018278), and performed in accordance with the ethical standards as laid down in the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Cell culture

Cell lines were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). The AML lines (Molm-13 and THP-1) was cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA) with 100 U/ml penicillin and 100 μg/ml streptomycin, and 293T and hFOB1.19 were all cultured in DMEM medium containing 10% FBS with 100 U/ml penicillin and 100 μg/ml streptomycin.

The cell viability assessment of H4-PEG-Glu

The cytotoxicity of **H4-PEG-Glu** was investigated by Cell counting kit-8 (CCK-8) assays. The cell viability was measured using AML lines (Molm-13 and THP-1) and normal cells (293T and

hFOB1.19) for 24 h incubation. The cells were seeded in a 96-well plate (around 5000 cells per well). After 12 h, the medium was substituted with the fresh medium contained **H4-PEG-Glu** with different concentrations. Followed by incubation for another 24 h, then Cell counting kit-8 (CCK-8) assays was performed for measuring the cell viability. All samples were performed in triplicate to ensure accuracy.

The photothermal conversion efficiency of H4-PEG-Glu

The photothermal conversion efficiency of H4-PEG-Glu was calculated according to the previous reported methods[4] with the following equation:

$$\eta = \frac{hS\Delta T_{max} - Q_s}{I(1 - 10^{-A808})}$$

where h refers to the heat transfer coefficient, S means the container surface area, and hS = mc/τ. m refers to the solution mass, c means the specific heat capacity of the solution, and τ is obtained from the cooling process and the corresponding time. ΔT_{max} is the difference between the equilibrium and the ambient temperature. Q_S is the heat dissipated from light absorbed by the centrifuge tube containing PBS, *I* and A_{808} refers to the laser power and the absorbance at 808 nm, respectively.

Trypan Blue exclusion test of cell viability

Using 10 μl each of cell suspension and 0.4% trypan blue, mixed and added to a cell counting chamber (Nexcelom Cellometer), then calculated cell number and viability using a Cellometer Mini Bright Field Cell Counter (Nexcelom Cellometer Mini).

Isolation of peripheral blood mononuclear cell (PBMC)

The peripheral blood was collected and diluted, and slowly added to the upper layer of Ficoll-Hypaque (Tianjin Haoyang). Density gradient centrifuge for isolating PBMC.

Flow cytometry

Molm-13 and THP-1 cells were seed on 6-well plates with 4 ×10^5 cells/well and cultured overnight. Then **H4-PEG-Glu** (20 μM) in fresh cell medium was replaced and incubated with cells for another 24 h. With or without 808 nm laser irradiation (1.2W/cm^2, 5 min), cells were centrifuged and resuspended in PBS with 20% FBS for 15 min at 4 °C refrigerator to block Fc receptors. The cells suspensions were incubated with the Annexin V-FITC for 10 min, after rinsed with PBS, all samples were measured and analyzed with FACS Canto II cytometer (Beckman Cytexpert). All samples were performed in triplicate to ensure accuracy.

AML Patients and AML patient derived PDX mouse models

All AML samples were acquired under the Institutional Ethical Committee of the Zhongnan Hospital of Wuhan University, China - approved protocol. All patients gave written informed consent. B-NDG mice (5-6 weeks old, 18-20g, female) were purchased from Beijing Biocytogen. All mice were fed following the institutional and national guidelines for the care and use of laboratory animals. Animal study protocol was approved by the Review Board of Zhongnan Hospital of Wuhan University. $CD34^+$ cells were isolated from AML patients. Before transplantation, B-NDG mice were given a χ-ray (1.0 Gy) irradiation for the whole body, followed by an intravenous injection of harvested AML patient $CD34^+$ cells (1.5 × 106 per mouse) within 24 h irradiation. Peripheral venous blood samples were collected at days 9 post-transplant. The bone marrow smear and blood smear were stained via Wright's staining and observed through an oil immersion lens microscope (OLYMPUS BX41) to measure the proportion of leukemia cells and evaluate whether the AML patient derived PDX mouse models were successfully constructed. The mice were randomly assigned to three groups.

The cell uptake assessment of H4-PEG-Glu and H4-PEG2K

For cell uptake study, the AML Molm-13 cells (1 ×10^5 cells per well) and THP-1 cells (1 ×10^5 cells per well) were cultured in six well plates with RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA), 100 IU mL^{-1} penicillin, 100 μg mL-1 streptomycin for 12 h. and substituted with the fresh medium contained **H4-PEG-Glu** (20 μM) and

H4-PEG2K (20 μM). Followed by incubation for different time, and the suspension cells were collected. Then the cells were washed twice with PBS and the precipitate of cells were collected for NIR-II imaging. And the cellular uptake ability of normal cells (293T and hFOB1.19) were conducted under the similar procedure.

Laser confocal microscopy

Molm-13 and THP-1 cells were cultured with different treatments for 24 h, stained with Mito-Tracker Green (5 μM) for 10 min, then cells were harvested and washed by PBS. Add 500 μL of 4% paraformaldehyde (pH 7.4) to the cell pellet, mix thoroughly and incubate for 10 min at 37 ℃ followed by centrifugation. Drop cells onto the slide. Mitochondrial localization marker and Mitochondrial membrane potential were detected using Mito-Tracker Green (Beyotime, China) and Mitochondrial membrane potential assay kit with JC-1 (Beyotime, China) according to the manufacturer's instructions and the literature procedure.[2] The change of cells was observed using laser confocal.

***In Vivo* NIR-II Fluorescence Imaging.**

All NIR-II fluorescent images were collected using a NIR-II imaging system with the indium-gallium-arsenide (InGaAs) camera (Princeton Instruments). The excitation light source was an 808 nm diode laser. The laser power density was 90 mW cm^{-2} with a 1000 nm long-pass filter during *in vivo* imaging. The mice were anesthesized by intraperitoneal injection of pentobarbital sodium solution (50 mg kg^{-1}) during the NIR-II imaging. Then the probe **H4-PEG-Glu** (200 μg, 1 μg/μL) was administrated to AML patient derived PDX mouse models through tail vein, After injection, the mice were mounted in the prone position beneath the laser for imaging at various time points (3 h, 6 h, 9 h).

$CD34^+$ cells preparation

The patient's PBMC were collected and filtered through a 70-μm cell strainer (BD Falcon). And $CD34^+$ cells were purified from PBMC of AML patients using MACS CD34 kit (Miltenyi Biotec) according to the manufacturer's instructions.

Biochemical index evaluation

ICR female mice (n= 6, three per group) treated with PBS and H4-PEG-Glu (200 μ g), respectively. Blood samples were collected from eyeball after 7 day post-injection. The blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (CREA) were performed by an automatic biochemical analyzer to assess liver and kidney functions.

Statistical analysis

All of the data were expressed as the mean ± SD. Differences between two groups were analyzed by a two-tailed Student's test. These analyses were carried out using SPSS 12.0. *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

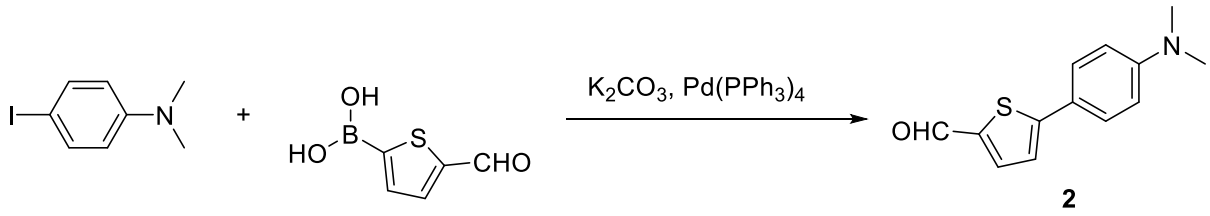
Synthesis

Synthesis and characterization of intermediate **1**, **2**, **H4**, **PEG-Glu**, **H4-PEG-Glu, H4-PEG2K**

The synthesis of intermediate 1

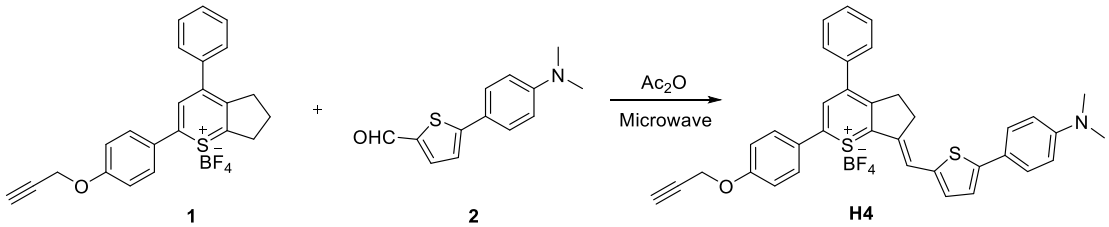
Intermediate **1** was obtained according to our previous reported literature.[1]

The synthesis of intermediate 2



A solution of 4-Iodo-N,N-dimethylaniline (1.57 g, 6.35 mmol) in toluene/ethanol (30 mL/30 mL) was treated with (5-formylthiophen-2-yl)boronic acid (1.19 g, 7.62 mmol), tetrakis(triphenylphosphine) palladium (0) (0.37 g, 0.3175 mmol) and 2M K_2CO_3 (6 mL).The mixture was stirred at 85 ºC under N_2 atmosphere for 3 h. The reaction was monitored by TLC. After reaction, the mixture was cooled to 25 ºC and poured into 100 ml water. Then the resulting mixture was extracted with DCM (50 mL x3). The organic phase was washed with brine and dried with anhydrous Na_2SO_4 and concentrated to give a crude product. The crude product was then purified by column chromatography on silica gel to give the desired product **2** (1.2 g, yield 55.6%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.84 (s, 1H), 7.70 (d, *J* = 3.9 Hz, 1H), 7.58 (d, *J* = 8.7 Hz, 2H), 7.26 (d, *J* = 3.9 Hz, 1H), 6.73 (d, *J* = 8.7 Hz, 2H), 3.04 (s, 6H). ^{13}C NMR (101 MHz, Chloroform-d) δ 182.6, 156.2, 151.2, 140.2, 138.2, 127.6, 121.6, 120.9, 112.2, 40.3. HRMS calcd for $C_{13}H_{13}NNaOS^+$([M+Na]$^+$): 254.0616, found:254.0632

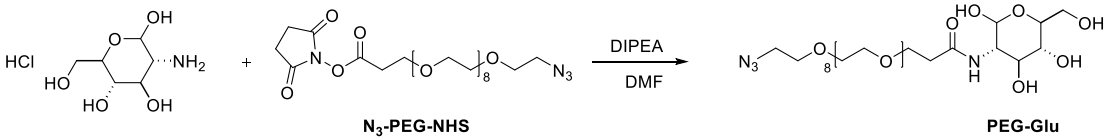
The synthesis of H4



A mixture of compound **1** (30 mg, 0.07 mmol) and compound **2** (24 mg, 0.1 mmol) in 10 mL tube sealing was dissolved in acetic anhydride (3 mL). The mixture was heated at 75 ºC under microwave for 2 h. After reaction, the mixture was cooled to room temperature and added Et_2O to form precipitate. And the precipitate was further purified by column chromatography on silica gel to give the desired product **H4** (25 mg, yield 52%). ^{1}H NMR (400 MHz, Acetonitrile-*d*3) δ 8.12 (s, 1H),

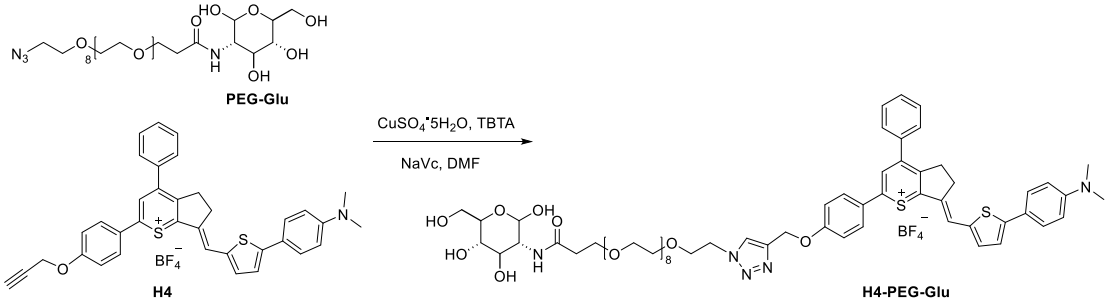
7.95 (s, 1H), 7.91 (d, *J* = 8.7 Hz, 2H), 7.70 (m, 2H), 7.67 (m, 3H), 7.58 (d, *J* = 3.8 Hz, 1H), 7.56 (d, *J* = 8.7 Hz, 2H), 7.44 (d, *J* = 4.0 Hz, 1H), 7.25 (d, *J* = 8.8 Hz, 2H), 6.68 (d, *J* = 8.8 Hz, 2H), 4.91 (d, *J* = 2.4 Hz, 2H), 3.41 (m, 2H), 3.27 – 3.09 (m, 2H), 2.95 (s, 6H), 2.92 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (101 MHz, Acetonitrile-*d*3) δ 169.7, 162.2, 158.8, 156.0, 152.1, 151.9, 149.1, 144.7, 138.8, 138.0, 134.1, 131.8, 130.4, 129.9, 129.7, 128.0, 127.9, 124.2, 123.8, 121.5, 120.9, 117.1, 112.9, 79.0, 78.7, 56.9, 32.8, 30.4, 21.3. HRMS calcd for $C_{36}H_{30}NOS_2^+$([M-BF_4]$^+$): 556.1763, found: 556.1784

The synthesis of PEG-Glu



(+)-D-glucosamine hydrochloride (2.35 mg, 0.01 mmol) and N_3-PEG_8-NHS (6 mg, 0.01 mmol) was dissolved in 1 mL DMF. Then DIPEA (14 mg, 0.1 mmol) was added. The resulting mixture was stirred overnight at 25 °C. After reaction, Et_2O was added into the mixture and cooled under -80 °C and precipitate was formed. Then centrifuge to remove the Et_2O to obtained the crude product **PEG-Glu**. The product was used directly into next step without further purification.

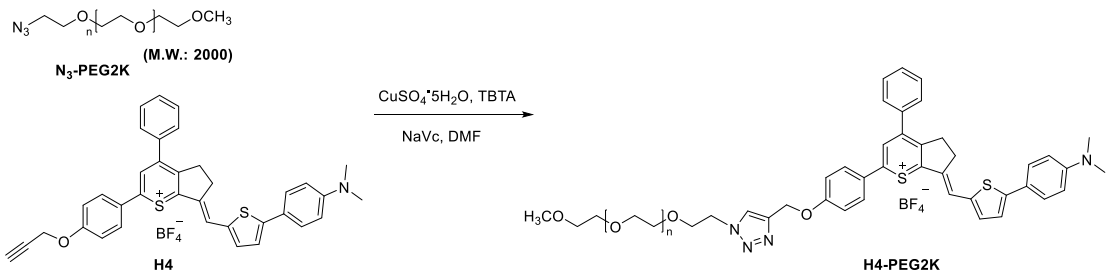
The synthesis of H4-PEG-Glu



To a solution of **H4** (15 mg, 0.023 mmol) and **PEG-Glu** (29 mg, 0.046 mmol) in 1mL anhydrous DMF was added a catalytical amount of $CuSO_4$•$5H_2O$, TBTA and VcNa. The mixture was stirred at 25 °C for 2 h. The reaction was monitored by HPLC. After reaction, Et_2O was added into the mixture and cooled under -80 °C and precipitate was formed. The precipitate was purified by HPLC (eluent: water/acetonitrile; containing 0.1% TFA, from 5 to 95%) to obtain **H4-PEG-Glu** (15.5 mg, yield

52.5%), which was characterized by MALDI-TOF-MS. $C_{61}H_{78}N_5O_{15}S_2^+$ ([M-BF4]+): 1184.4930, found: 1184.1915.

The synthesis of H4-PEG2K



To a solution of **H4** (5 mg, 0.0077 mmol) and **N_3-PEG2K** (15.4 mg, 0.023 mmol) in 200 mL anhydrous DMF was added a catalytic amount of $CuSO_4$•$5H_2O$, TBTA and VcNa. The mixture was stirred at 25 ºC for 24 h. The reaction was monitored by HPLC. After reaction, the mixture was diluted with 1.5 mL distilled water and was further purified by HPLC (eluent: water/acetonitrile; containing 0.1% TFA, from 5 to 95%) to obtain **H4-PEG2K** (10 mg, yield 52%), which was characterized by MALDI-TOF-MS.

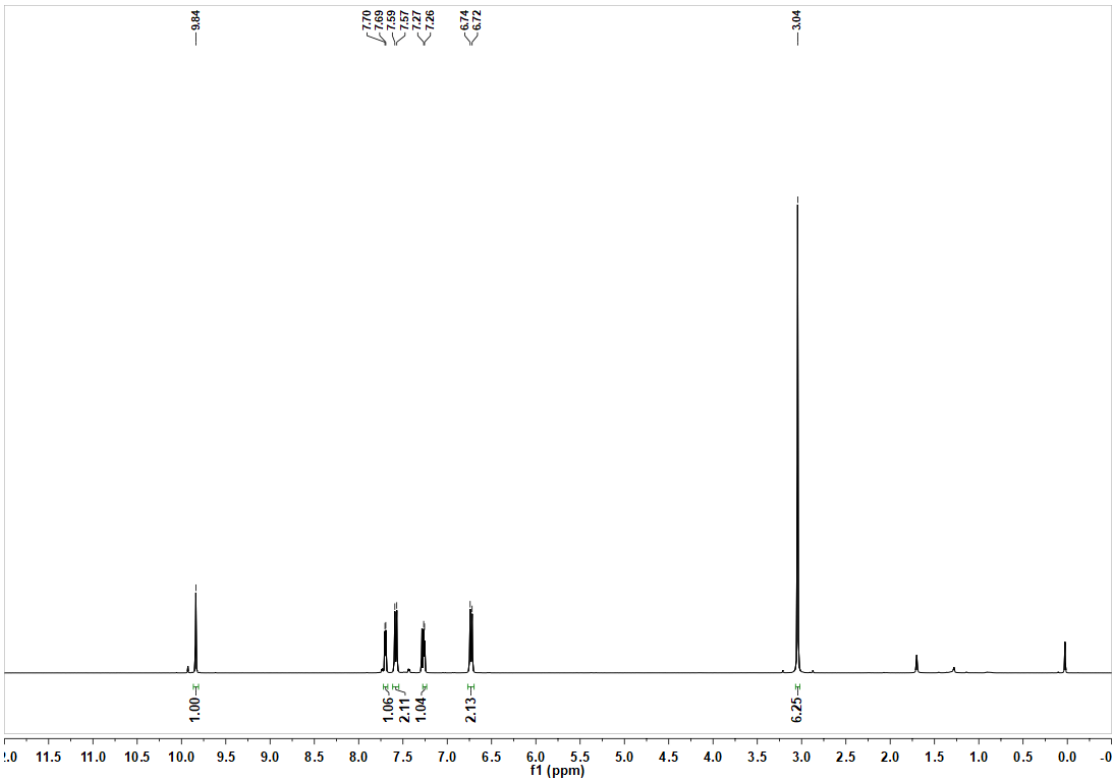


Fig. S1. ^{1}H NMR of compound **2**

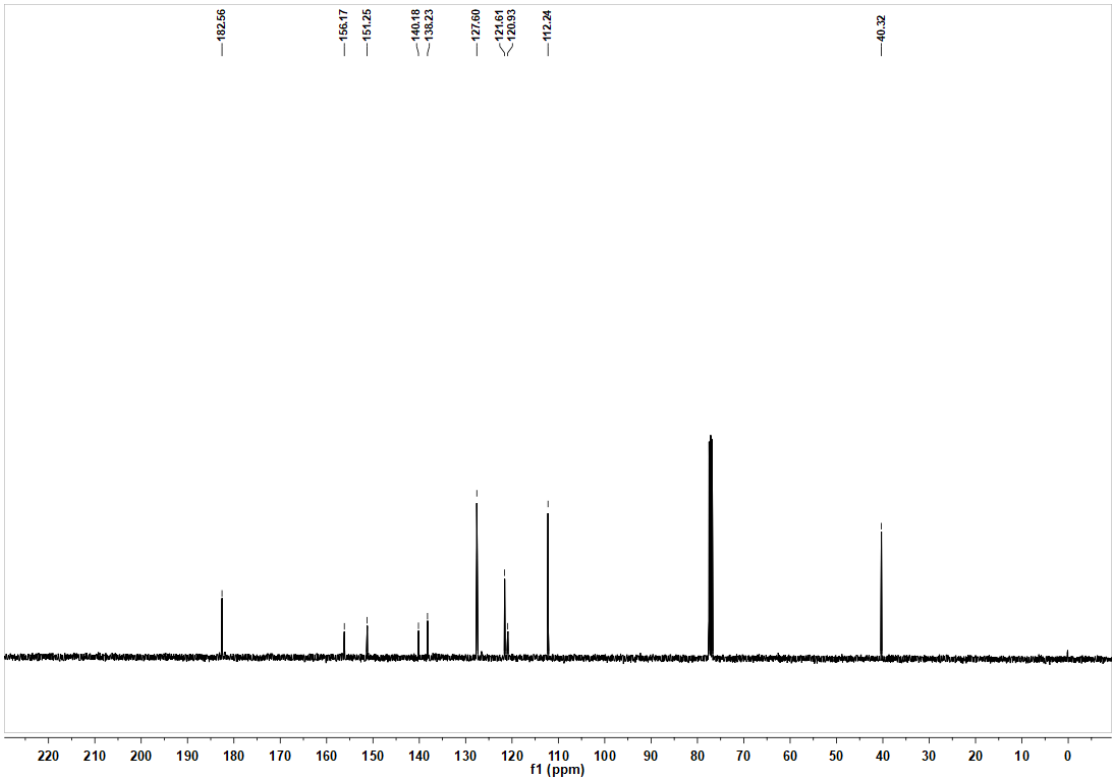


Fig. S2. ^{13}C NMR of compound **2**

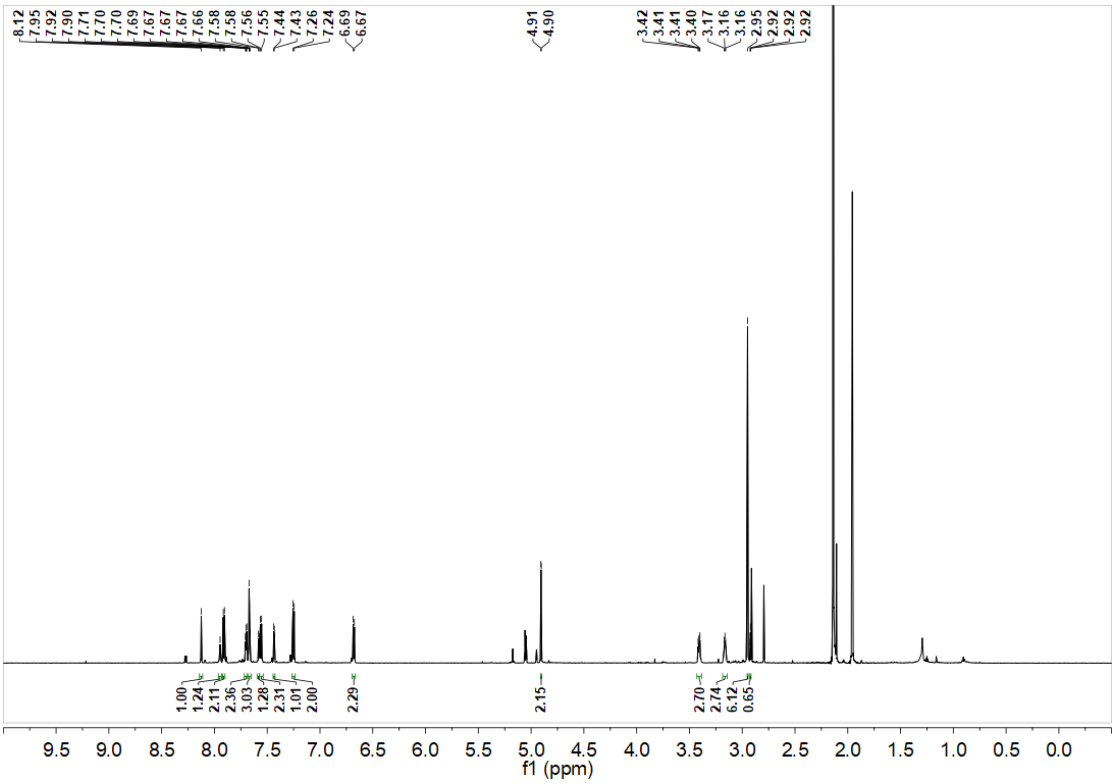


Fig. S3. ^{1}H NMR of compound **H4**

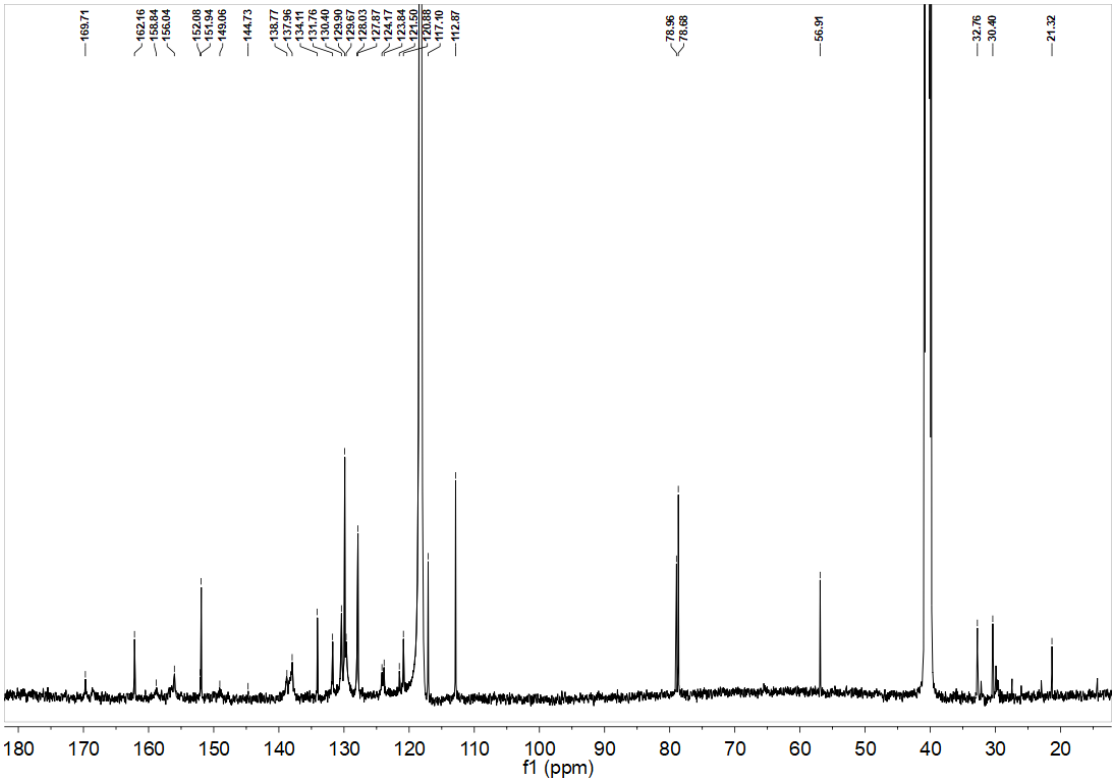


Fig. S4. ^{13}C NMR of compound **H4.**

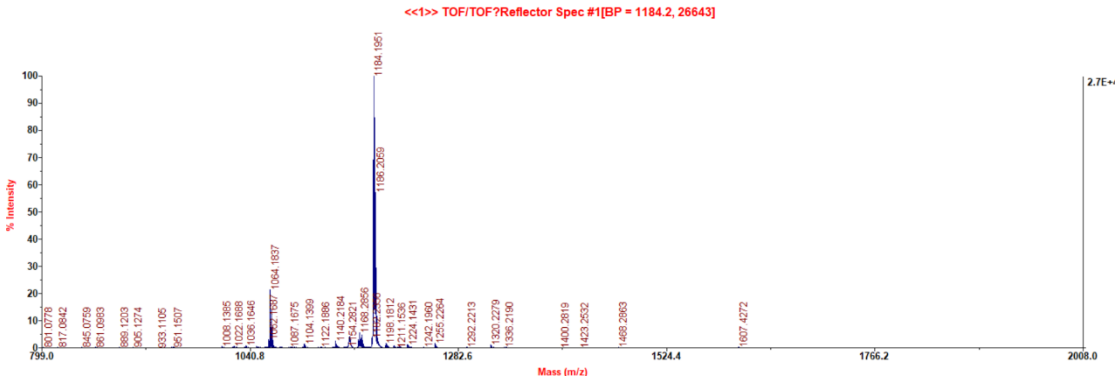


Fig. S5. The MALDI-TOF-MS of **H4-PEG-Glu.**

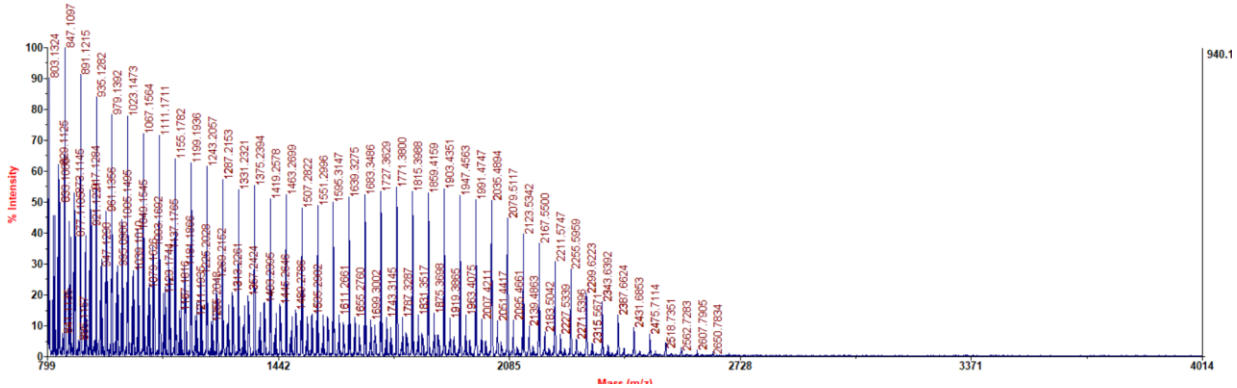


Fig. S6. The MALDI-TOF-MS of **H4-PEG2K.**

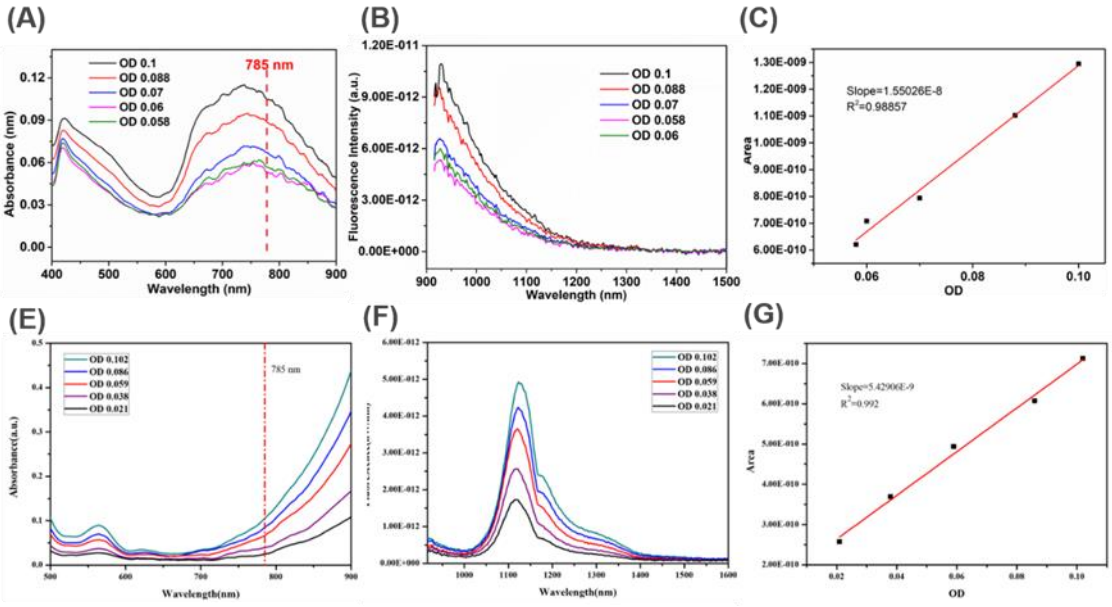


Fig. S7. Absorbance (A, E) and fluorescence (B, F) spectra of **H4-PEG-Glu** in Serum (A, B), and **IR-26** in DCE (E, F). The slope of **H4-PEG-Glu** in Serum (C) and **IR-26** in DCE (G).

Fluorescence quantum yield measurements of **H4-PEG-Glu** in Serum were calculated according to a standard equation in the previous literature [3]. The equation was below:

$$QY_{sam} = QY_{ref} \times \frac{S_{sam}}{S_{ref}} \times \left(\frac{n_{sam}}{n_{ref}}\right)^2$$

Where QY_{sam} is the QY of **H4-PEG-Glu**, QY_{ref} is the quantum yield of IR-26 (~ 0. 5%), S_{sam} and S_{ref} are the slopes obtained by linear fitting of the integrated fluorescence intensity of **H4-PEG-Glu** (1000-1600 nm) and IR-26 (1000-1600 nm) against the absorbance at 785 nm. n_{sam} and n_{ref} are the refractive indices of their respective solvents (Serum ≈ H_2O:1.33 and DCE: 1.42)

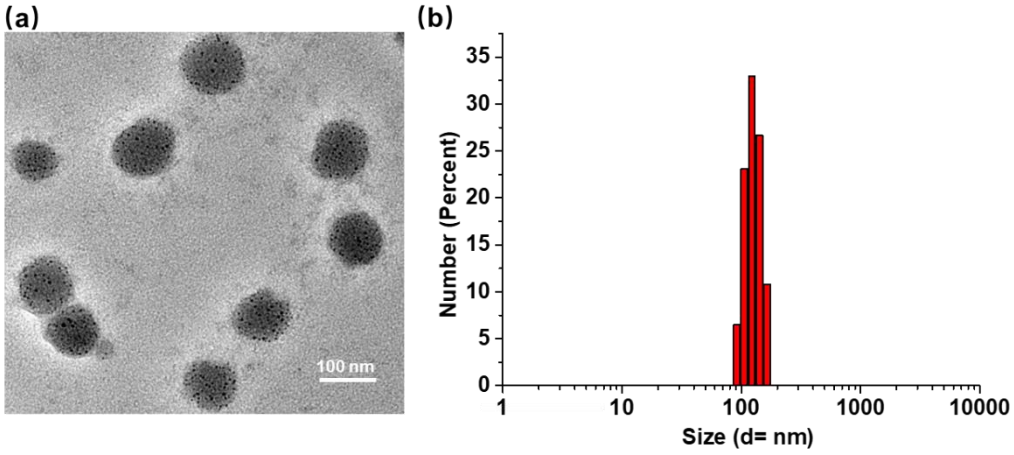


Fig. S8. The TEM and DLS images of **H4-PEG-Glu**, scale bar: 100 nm

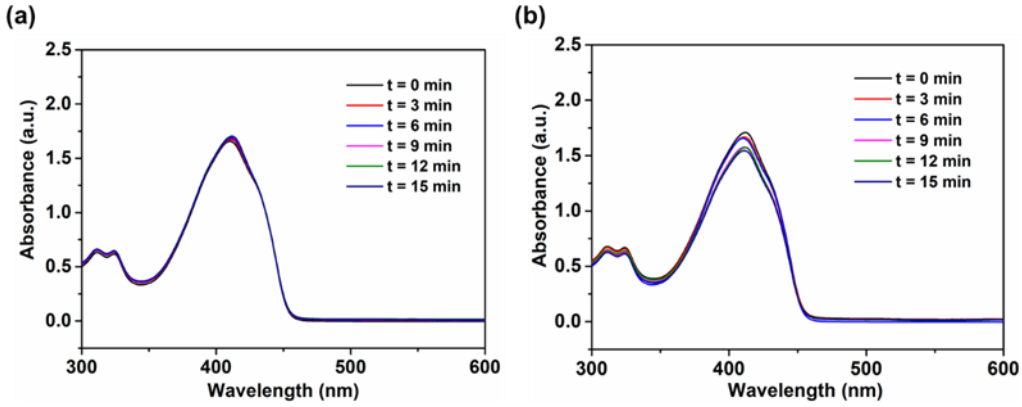


Fig. S9. Time-course absorption spectra of DPBF solution under 808 nm laser irradiation at a power density of 1.2 W/cm^2 (a) alone or (b) with **H4-PEG-Glu** (50 μg/mL).

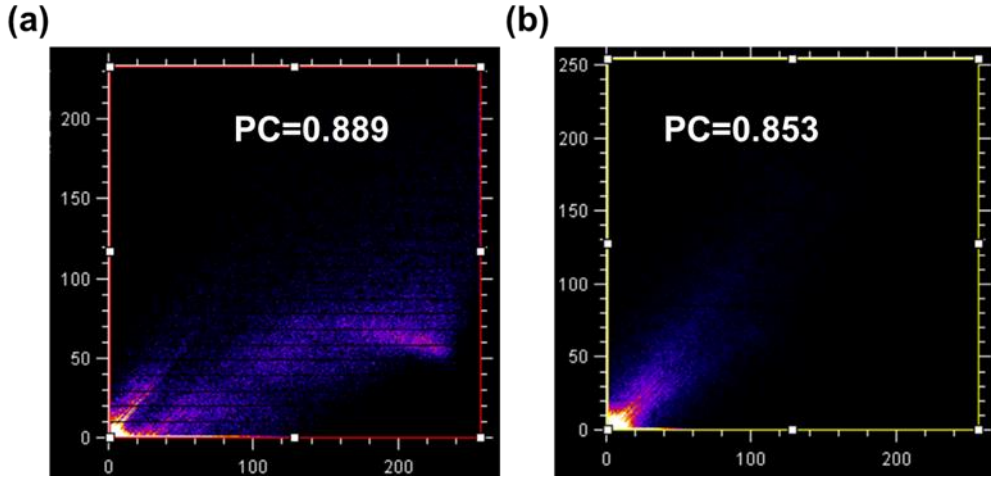


Fig. S10. (a) Colocalization scatterplots of **H4-PEG-Glu** and Mito-tracker Green in THP-1 cells, PCC = 0.889, OLC= 0.902. (b) Colocalization scatterplots of **H4-PEG-Glu** and Mito-tracker Green in Molm-13 cells, PCC = 0.853, OLC= 0.874.

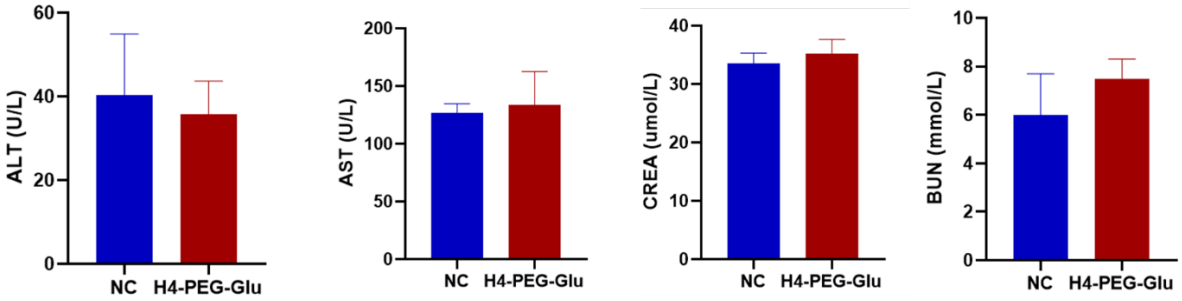


Fig. S11. The comparison of blood biochemical index (ALT、AST、CREA、BUN) of **H4-PEG-Glu** treated and PBS treated mice after 7 days.

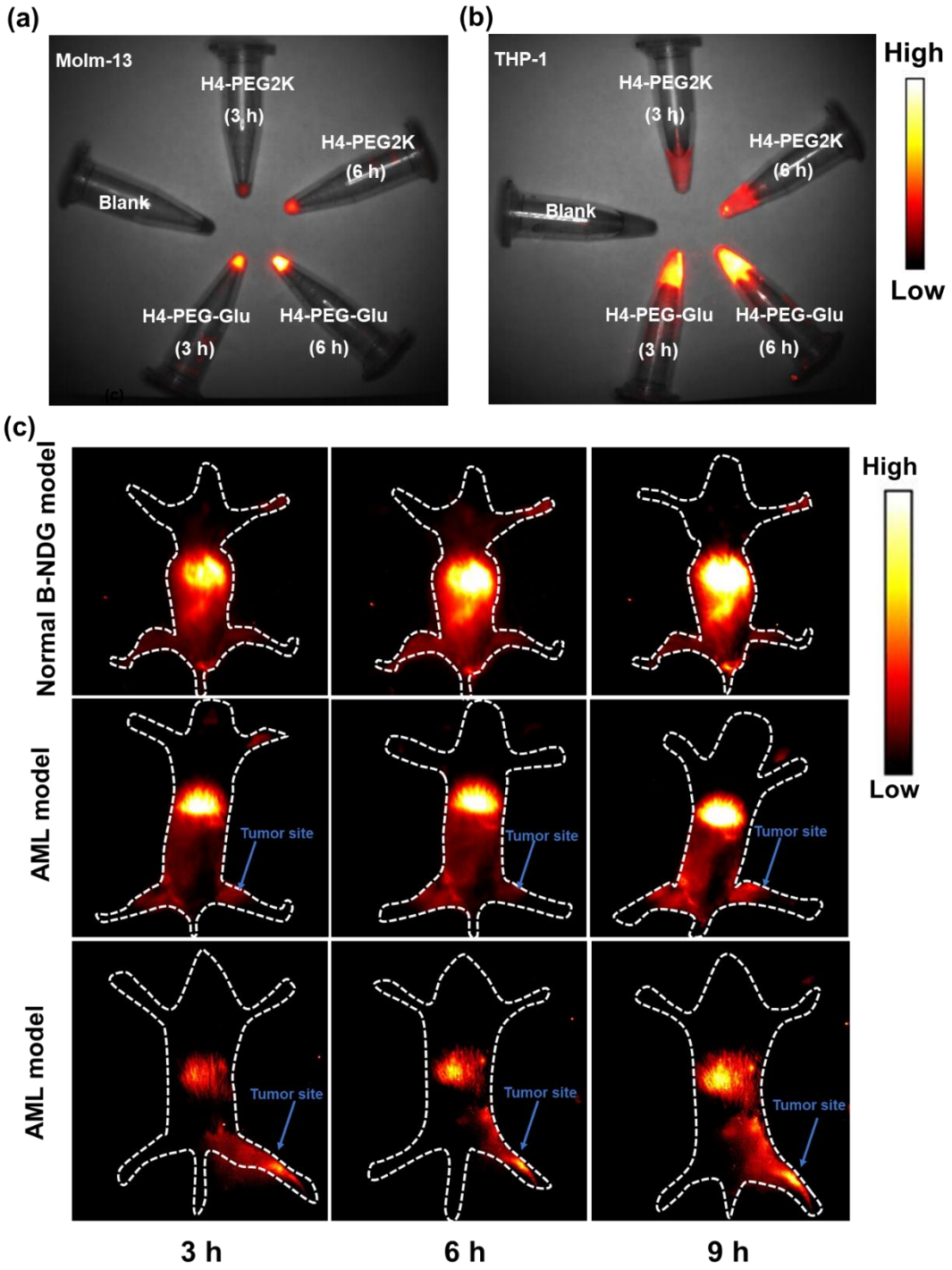


Fig. S12. (a) The NIR-II signals of Molm-13 cells by **H4-PEG2K** (100 μL, 1 mg/mL) and **H4-PEG-Glu** (100 μL, 1 mg/mL) under 808 nm excitation (1000 LP and 100 ms); (b) The NIR-II signals of THP-1 cells by **H4-PEG2K** (100 μL, 1 mg/mL) and **H4-PEG-Glu** (100 μL, 1 mg/mL) under 808 nm excitation (1000 LP and 100 ms); (c) The NIR-II images of **H4-PEG2K** (100 μL, 1 mg/mL) in normal B-NDG model and AML model (the first and second column) and **H4-PEG-**

Glu (100 μL, 1 mg/mL) in AML patient derived PDX mouse models (n= 3) (the last column) with different time points.

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