Supporting information

Photo-stable and reversible pH-response nano-agent based on NIR phenazine dye for photoacoustic imaging-guided photothermal therapy

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Materials and measurements

Dichloromethane (DCM) was refluxed with calcium hydride and distilled before use. Ethanol (EtOH) were pre-dried over 4 Å molecular sieves before use. All other reagents and reactants were purchased as commercial products from Energy Chemical or Sigma-Aldrich and used as received without further purification.

$^1\text{H}$ NMR and $^{13}\text{C}$ NMR spectra were recorded on a Bruker AM 400 MHz spectrometer, and tetramethylsilane (TMS) were used as an internal standard. Electrospray ionization and time-of-flight analyzer (ESI-TOF) mass spectra were determined using a Waters Micromass LCT mass spectrometer. Absorption spectra were recorded on a Varian Cary 500 UV-vis spectrophotometer. The size distribution of the nanoparticles was measured using an ALV-5000 laser light scattering spectrometer (DLS). MTT data was measured by microreader (Model 680, Bio-Rad).

The PH-CHO and compound 1 was synthesized according to previous report.[1]

MDA-MB-231 cells (human breast cancer cells) were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. FBS (fetal calf serum) and DMEM (dulbecco's modified eagle medium) were procured from Gibco. Nude BALB/c mice were purchased from the Animal Experiment Centre of Shanghai Cancer Institute. All of the animal experimental procedures were performed according to the protocols approved by the Animal Care and Use Committee of the Shanghai Cancer Institute.

Scheme S1 Synthetic routes of PIOH.

Synthesis of PIOH. PH-CHO (196 mg, 0.56 mmol) and 3H-Indolium, 1-(3-hydroxypropyl)-2,3,3-trimethyl-, iodide (205 mg, 0.59 mmol) was dissolved in 15 mL acetonitrile. Then, a drop of piperidine was added into the solution. Subsequently, the mixture was heated to 80 °C under the protection of argon for 10 hours. PIOH (54.3 mg, 17.6%) was purified by silica gel chromatography (CH$_2$Cl$_2$/alcohol, v: v = 12:1) then recrystallized in basic aqueous solution. $^1\text{H}$ NMR (400 MHz, CDCl$_3$) δ 9.57 (s, 1H), 7.16 (t, $J = 7.3$ Hz, 1H), 7.08 (d, $J = 7.0$ Hz, 1H), 7.04 (d, $J = 8.1$ Hz, 1H), 6.84 (t, $J = 7.3$ Hz, 1H), 6.78 (d, $J = 7.5$ Hz, 1H), 6.66 (d, $J = 7.8$ Hz, 1H), 6.64 – 6.58 (m, 2H), 6.42 (s, 1H),
6.25 (d, $J = 7.2$ Hz, 2H), 5.97 (d, $J = 16.4$ Hz, 1H), 3.79 – 3.51 (m, 4H), 3.49 – 3.38 (m, 4H), 1.66 (ddd, $J = 16.6, 11.6, 7.6$ Hz, 6H), 1.52 – 1.45 (m, 4H), 1.32 (s, 3H), 1.25 (s, 3H), 1.06 – 1.01 (m, 6H). $^{13}$C NMR (101 MHz, DMSO-$d_6$+CF$_3$COOD) δ 190.07, 170.15, 142.89, 141.64, 141.59, 141.24, 134.39, 129.97, 129.60, 129.55, 128.88, 128.01, 122.90, 113.85, 111.49, 111.13, 109.98, 106.95, 78.40, 66.29, 51.24, 44.41, 42.93, 32.87, 31.04, 28.95, 26.24, 20.46, 20.41, 19.28, 19.25, 13.79, 13.67. MS (ESI, m/z): [M]$^+$ calcd for C$_{36}$H$_{44}$N$_3$O$_2$: 550.3434; found: 550.3436.

**Calculation of pKa.**

pKa of PIOH was calculated by formula:

$$\log\left(\frac{A_{\text{max}} - A}{A - A_{\text{min}}}\right) = \text{pH} - \text{pKa}$$

![Graph showing the relationship between pH and log transformation of (Amax - A)/(A - Amin)](image)

**Synthesis of PIOH-NPs**

1.0 mg PIOH, 5.0 mg cholesterol and 20.0 mg soya bean lecthin was dissolved in 1.0 mL dichloromethane. The solution was emulsified with 3.0 mL ultrapure water. Then the dichloromethane was removed in vacuo.

**MTT assay**

MDA-MB-231/MCF-7 cells were seeded to 96 well plate ($1 \times 10^4$ per well), and hatched in DEME culture medium (contain 10% fetal calf serum) for 24 hours. PIOH-NPs were attenuated with culture medium to get different concentration solution (2.25 μM, 4.5 μM, 9 μM, 18 μM, 36 μM) and replacing previous medium. Cells were hatched in medium with PIOH-NPs for another 24 h. The methyl thiazolyl tetrazolium (MTT) assay was utilized to evaluate the cytotoxicity of PIOH-NPs.
Subsequently, cells were cultured with MTT (150 μL, 0.5 mg/mL) for 4 hours. The absorbance of each well was measured under a 490 nm laser by a microplate reader (Bio-Rad Laboratories Inc, Hercules, CA, USA).

The relative growth rate of the cells (RGR) was determined by the following equation:

\[ \text{RGR} \% = \left( \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \right) \times 100\% \]

**Photothermal cytotoxicity of PIOH-NPs**

MDA-MB-231 cells were seeded to 96 well plate (1×10^4 per well), and hatched in DEME culture medium (contain 10% fetal calf serum) for 24 hours. Then the medium was replaced with PIOH-NPs solutions (pH = 6.0 and pH = 7.4) and exposed to laser (808 nm, 1 W/cm²) for different time. Subsequently, cells were hatched in fresh medium for another 24 h. The methyl thiazolyl tetrazolium (MTT) assay was utilized to evaluate the cytotoxicity of PIOH-NPs. Subsequently, cells were cultured with MTT (150 μL, 0.5 mg/mL) for 4 hours. The absorbance of each well was measured under a 490 nm laser by a microplate reader (Bio-Rad Laboratories Inc, Hercules, CA, USA).

Photothermal cytotoxicity of PIOH-NPs was also measured by AM-PI assay via fluorescence microscope imaging. The experiment was divided into three groups, control group and experiment group. MCF-7 cells were seeded in petri dishes at a density of 5×10^4 cells per dishes. In experiment group, cells were treated by PIOH-NPs (30 μM) at pH = 7.4 and pH = 6.0, then exposed to 808 nm laser (1 W/cm²) for 10 min, while in control group, cells were exposed to 808 nm laser without NPs. Cells were further co-stained with 10 μg Calcine AM (AM) and 200 nM prodidium iodide (PI).

**Photoacoustic Imaging**

PIOH-NPs solution (300 μL, 0.6 mM) were injected in tail vein. Then the tumor of nudes were imaged by VEVO Lazer at different time point (0h, 3h, 7h, 24h).

**In vivo live imaging for PIOH-NP distribution**

The mice were divided into three groups randomly (n = 3). Free Dir and Dir-NPs were administered by intravenous injection when the volume of the tumor reached 100 mm³. Then, at 1, 4, 24, 48 and 72 h post-injection, the nude mice were anesthetized by Isoflurane, and fluorescence indicating the PIOH-NP was observed using in vivo imaging device (LB983, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). After that, the nude mice were separately sacrificed. The heart, liver, spleen, lung, kidney, tumor or subcutaneous tissue were harvested and observed.

**Tumor growth inhibition study in vivo**

The animal models were Balb/c background female nude mice purchased in a pathogen-free animal environment.
room of Fu Dan University (Shanghai, China). The BALB/C mice (4–6 weeks old, 18–22 g, Female) were obtained from Shanghai SLAC Laboratory Animal (Shanghai, China). The SMMC-7721 subcutaneous hepatic cancer tumor model was created as described by previous reports. Briefly, the 7721 cells were subcutaneously injected (1.5 × 10^6, 100 μl in PBS) in the right forelimb of the nude mice. The mice were randomly divided into 6 groups (n=5) until the tumor volume grew to approximately 100 mm^3. Then the mice were randomly divided into 5 groups and treated with PBS, Laser, PIOH + Laser, PIOH-NP and PIOH-NP + Laser through intravenous injection. The body weight and tumor size of each mouse were measured at day 3, 5, 8 and 16. Afterwards, the relative tumor volumes were obtained by equations at following:

Tumor volume (V) \( V = \frac{ab^2}{2} \)

Relative tumor volume (RTV) = \( V_t / V_C \)

Where \( V_t \) and \( V_C \) are the tumor volume after the different treatment and the volume of the untreated tumor in PBS, respectively. And a and b represent long and short diameters, respectively, of the tumor.

**In vivo safety evaluation of PIOH and PIOH-NP**

The treated nude mice were sacrificed at day 16. Serum samples were collected by retro-orbital bleeding and measured to assess hepatic and renal damage. Tissues from the heart, liver, spleen, lung, kidney and tumor were subsequently analyzed by HE staining sections.

![Fig. S1](image)

**Fig. S1** 1H NMR spectrum of PIOH in CDCl3.
Fig. S2 $^{13}$C NMR spectrum of PIOH in DMSO-$d_6$+CF$_3$COOD.

Fig. S3 High resolution mass spectrum of PIOH.
Fig. S4 fluorescence spectra in different pH buffer solution (DMSO: BR buffer=3:7, 10 μM),
\[ \lambda_{ex}=525 \text{ nm} \]

Fig. S5 Reversible change of absorbance at 690 nm of PIOH between pH 8.5 and pH 2.2
Fig. S6 $^1$H NMR spectra of PIOH in acid (DMSO-$d_6$+CH$_3$COOH) and basic (DMSO-$d_6$+NaOD in D$_2$O) surrounding.

Fig. S7 High resolution mass spectrum of PIOH in alkaline and acidic solution.
Fig. S8 SEM image of PIOH-NPs

Fig. S9 (a) DLS size distribution of PIOH-NPs in pure water. The diameter and PDI of PIOH-NPs at different days (b) and in different pH buffers (c).

Fig. S10 Absorbance (A) and absorbance at 690 nm (B) of PIOH-NPs in different pH buffer solution. Photography of PIOH-NPs in different pH buffer solution.
Fig. S11 Cell viability of different concentration PIOH-NPs.

Fig. S12 Photography of mice in every group at different time point.
Fig. S13 Biosafety evaluation of PIOH-NP. (A) HE sections of main organs from treated groups. Liver function evaluation presented by (B) Serum ALT (U/L) detection and (C) Serum AST (U/L) detection from different treated groups. Kidney function evaluation presented by (D) Serum Cr detection from different treated mice.