A pH-responsive zinc (II) metalated porphyrin for enhanced photodynamic/photothermal combined cancer therapy

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ABSTRACT The acidic tumor microenvironment is triggered by glycolysis in hypoxic condition, which can motivate the pH-responsive system to build certain triggers for efficiently tumor-targeted phototherapy. Additionally, the metalated porphyrin structures are widely studied in biomedical applications due to the favorable properties of high singlet oxygen quantum yield as well as strong fluorescence imaging ability. Herein, a pH-responsive zinc (II) metalated porphyrin (P-4) was designed and synthesized for amplifying cancer photodynamic/photothermal therapy with excellent fluorescence quantum yield (67.4%), superb singlet oxygen quantum yield (84.3%) and desired photothermal conversion efficiency (30.0%). *In vitro*, the self-assembled P-4 nanoparticles can specifically target to lysosome subcellular site and realize protonated process of dibutanaminophenyl (DBAP) groups with high photo toxicity. Under single 660 nm laser illumination, the tumor can be ablated completely with no side effects *in vivo*. This work demonstrates that the pH-responsive P-4 nanoparticles provide a new avenue for highly efficient cancer combination therapy.

Keywords: pH-responsive, porphyrin, NIR absorbance, photodynamic therapy, photothermal therapy

INTRODUCTION

Photodynamic therapy (PDT) has captured great notice in malignant cancer therapy, in which, the photosensitizer around the cancer cells can absorb light and

transfer energy to the oxygen for producing reactive oxygen species (ROS), especially the singlet oxygen (¹O₂) to kill tumor cells [1,2]. On the other hand, photothermal therapy (PTT) agent can absorb light of specific wavelength and undergo non-radiative decay with heat release, resulting in irreversible cell damage, which has experienced explosive growth for cancer therapy in past decades [3]. However, for PDT, insufficient oxygen supply (hypoxia) [4,5] and inadequate selectivity with prolonged skin photosensitization significantly impede the therapeutic effect [6,7]. For PTT, the up-regulated expression of heat shock proteins greatly decreases the treatment effect and limits its application in clinic [8–10]. Hence, to overcome these obstacles, combining two different therapeutic modalities, such as PDT and PTT, into one system to exceed the individual outcome is a favorable method to achieve improved anticancer therapy efficacy in recent years [11–13]. Nonetheless, most of the combined therapeutic agents are required to be excited by different lasers with two wavelengths because of the mismatched absorbance from photothermal and photosensitizer functional groups, resulting in longer therapeutic time and higher treatment cost [14–16].

Moreover, the short ¹O₂ half-time (< 0.04 μs) with low diffusion depth (< 0.02 μm) [17,18], means that the targeted delivery of therapeutic agent to the tumor site is very important for the effective phototherapy [19]. In addition, the acidic tumor microenvironment (TME) is

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triggered by glycolysis in hypoxic condition. And the lysosome in tumor cells has a slightly acidic (pH 4.5–5.0) environment compared with the cytoplasm (pH 7.2), which is kept by vacuolar-type H^+ -ATPase [20,21]. Therefore, in recent years, pH-driven phototherapy agents have attracted significant interest to build certain triggers [22,23]. Unfortunately, most of these agents lack the selective localization in cancer cells or at tumor site, making the biological application incompatible [22,24,25].

Motivated by all above, we designed and synthesized a pH-responsive zinc (II) metalated porphyrin (P-4) for amplifying cancer PDT/PTT (Scheme 1). In self-assembled P-4 nanoparticles (NPs), the central chromophore porphyrin can be activated to produce abundant singlet oxygen as well as strong fluorescence, and the dibutaneaminophenyl (DBAP) groups with pH-responsive property can make the protonation in acidic TME for improving the 1O_2 quantum yield and photothermal conversion (PTC) efficiency. All experiments reveal the near-infrared (NIR) P-4 NPs targeting at lysosome sub-cellular site possess low dark toxicity, excellent phototoxicity in cancer cells, suggesting a desired photo ablation capability at tumor site *via* single laser illumination. Therefore, the as-prepared NPs can be potentially used as efficient agent for pH-responsive cancer photodynamic and photothermal synergetic therapy in clinic.

EXPERIMENTAL SECTION

Materials

Fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and other biological reagents were obtained from GIBCO. Green Lyso-Tracker, Mito-Tracker, ER-Tracker dyeing liquid and 4',6-diamidino-2-phenylindole (DAPI) were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (China). All other commercially available chemical agents were acquired from Sigma-Aldrich and used as received.

Characterizations

All the intermediate products and P-4 NPs were characterized by UV-vis-NIR spectrometer (Shimadzu, Japan), Fluorescence F-7000 spectrometer (HITACHI, Japan), 1H NMR, ^{13}C NMR (Nuclear magnetic resonance, Bruker DRX NMR spectrometer), matrix-assisted laser desorption/ionization time-of-flight mass microscopy (MALDI-TOF MS, Bruker autoflex speed MALDI-TOF), dynamic light scattering (DLS, Malvern Zeta Sizer) and

Scheme 1 Simplified representation of multi-imaging guided pH-responsive cancer PTT/PDT *in vitro* and *in vivo* with P-4 NPs.

transmission electron microscopy (TEM, JEM-2010FEF). Other apparatus included confocal fluorescence microscope (Olympus IX 70), inverted fluorescence microscope (Nikon, Japan), Flow Cytometry System, IR thermal camera (FLIR System, Inc., Wilsonville, OR, USA), optical fiber coupled 660-nm diode-laser and *in vivo* bioluminescence imaging system. All nude mice (five weeks aged, weight 18–20 g) were gained from Comparative Medicine Centre of Yangzhou University. Sprague-Dawley (SD) rats with specific-pathogen-free (SPF) (body weight 220 ± 10.0 g) were obtained from the Animal Center of Nanjing Medical University (NJMU, Nanjing, China). All procedures were performed in strict accordance with the relevant institutional guidelines and laws.

Synthesis of P-4

4-Butoxybenzaldehyde (1.5 g), and dipyrromethane (1.23 g) were added in dichloromethane (DCM, 300 mL) under N_2 atmosphere to remove oxygen in ultrasonic cleaner for 30 min. Next, trifluoroacetic acid (TFA, 0.5 mL) was slowly injected in dark for 3 h. Then 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 1 g) was added for another 30 min. At last, triethylamine (10 mL) was used to quench the reaction. The crude product was purified by chromatography on silica column (DCM/petroleum ether (PE), V/V = 1:1) to obtain purple P-1 (420 mg, yield: 8.7%). 1H NMR ($CDCl_3$, 400 MHz): δ 10.32 (s, 2H), 9.41 (d, $J=3.6$ Hz, 4H), 9.14 (d, $J=3.6$ Hz, 4H), 8.19 (d, $J=1.6$ Hz, 4H), 7.36 (d, $J=3.6$ Hz, 4H), 1.28 (s, 18H), -3.05 (s, 2H). MALDI-TOF MS: m/z calcd. for $C_{40}H_{38}N_4O_2$ 606.3; found 606.8.

P-1 (200 mg) and *N*-bromosuccinimide (NBS, 235 mg)

were dissolved in DCM (40 mL) under N₂ atmosphere to remove oxygen and reacted for 3 h. Then, the solvent was distilled and the residue was purified on silica column (DCM/PE, V/V = 1:1) to afford purple P-2 (240 mg, yield: 95%). ¹H NMR (CDCl₃, 400 MHz): δ 9.63 (d, *J* = 3.6 Hz, 4H), 8.90 (d, *J* = 3.6 Hz, 4H), 8.07 (d, *J* = 6.4 Hz, 4H), 7.32 (d, *J* = 6.4 Hz, 4H), 1.30 (m, 18H), −2.68 (s, 2H). MALDI-TOF MS: *m/z* calcd. for C₄₀H₃₆Br₂N₄O₂ 764.1; found 764.2.

P-2 (240 mg) and zinc acetate (58 mg) were mixed in DCM (90 mL) and methyl alcohol (10 mL) in dark to react for 3 h. At last, the solvent was removed and the residue was purified on silica column (pure DCM) to achieve purple P-3 (267.7 mg, yield: 98%). ¹H NMR (CDCl₃, 500 MHz): δ 10.22 (s, 1H), 9.79 (d, *J* = 5.0 Hz, 2H), 9.41 (d, *J* = 5.0 Hz, 2H), 9.09 (d, *J* = 5.0 Hz, 2H), 8.99 (d, *J* = 5.0 Hz, 2H), 8.02 (d, *J* = 10.0 Hz, 2H), 7.33 (d, *J* = 10.0 Hz, 2H), 1.28 (m, 18H). MALDI-TOF MS: *m/z* calcd. for C₄₀H₃₄Br₂N₄O₂Zn 827.0; found 827.1.

P-3 (50 mg), *N,N*-dibutyl-4-ethynylaniline (synthesized as described in the Supplementary information, 54 mg), trans-dichlorobis(triphenyl-phosphine)palladium(II) (40 mg) and CuI (20 mg) were added in THF (9 mL) and triethylamine (3 mL) under N₂ atmosphere in dark at 55°C for 6 h. Last, the solvent was removed and the crude product was purified on silica column (DCM/PE, V/V = 1:1) to attain deep green P-4 (55 mg, yield: 82%). ¹H NMR (CDCl₃, 400 MHz): δ 9.73 (d, *J* = 4.6 Hz, 4H), 8.92 (s, 4H), 8.11 (d, *J* = 8.5 Hz, 4H), 7.82 (d, *J* = 8.8 Hz, 4H), 7.32 (d, *J* = 8.5 Hz, 4H), 6.76 (d, *J* = 8.9 Hz, 4H), 4.31 (t, *J* = 6.5 Hz, 4H), 3.42–3.35 (m, 8H), 2.04–1.99 (m, 4H), 1.75–1.64 (m, 12H), 1.45 (dd, *J* = 15.0, 7.5 Hz, 8H), 1.15 (t, *J* = 7.4 Hz, 6H), 1.04 (t, *J* = 7.4 Hz, 12H) (Fig. S1); ¹³C NMR (CDCl₃, 126 MHz) δ 158.92, 151.97, 150.06, 148.85, 148.27–148.11, 135.43, 134.63, 132.96, 132.33, 130.73, 122.23, 112.73, 111.55, 109.72, 90.55, 68.06, 50.80, 31.61, 29.67, 20.37, 19.39, 13.99 (Fig. S2). MALDI-TOF MS: *m/z* calcd. for C₇₂H₇₈N₆O₂Zn 1,122.548; found 1,121.369 (Fig. S3).

Preparation and characterizations of P-4 NPs

P-4 NPs were acquired by the self-assembly method. Two milligram of P-4 (in 1 mL THF) was added into 10 mL of phosphate buffered solution (PBS, pH 7.4 and 5.0) at 25°C. During this process, P-4 molecules self-assembled into P-4 NPs. After self-assembling, THF was eliminated by nitrogen bubbling for 10 min.

The microstructure of P-4 NPs was characterized by TEM, and particle sizes of the NPs were detected by DLS. The UV-vis-NIR and fluorescence spectrum data were

measured by quartz cuvettes (optical path-length: 1 cm) in wavelength range of 300–1,100 nm and 700–900 nm, respectively.

For detection the fluorescence quantum yield of P-4 (in DCM), Indocyanine Green (ICG) in dimethyl sulfoxide (DMSO) ($\Phi_F(s) = 0.13$) was used as standard. The $\Phi_F(x)$ value was calculated by the following equation: $\Phi_F(x) = \Phi_F(s) \times (A_s/A_x) \times (F_x/F_s) \times (n_x/n_s)^2$, where *A* is the absorbance, *F* is the area under the fluorescence emission curve, *n* is the refractive index of the solvent, the subscripts X and S represent the sample and standard, respectively.

The singlet oxygen (¹O₂) generations of P-4 and P-4 NPs were measured *via* recording oxidation of diphenylisobenzofuran (DPBF, 2 × 10^{−5} mol L^{−1}, 60 s) and Singlet Oxygen Sensor Green (SOSG, 1.5 μg mL^{−1}, 9 min). The absorbance of DPBF at 416 nm and fluorescence intensity of SOSG at 531 nm were detected by UV-vis-NIR and fluorescence spectrophotometer with quartz cuvettes (optical path-length: 1 cm), respectively. The ¹O₂ quantum yield of P-4 was calculated by $\Phi_{\Delta(X)} = \Phi_{\Delta(MB)} \times (S_X/S_{MB}) \times (F_{MB}/F_X)$, in which the subscripts X and MB represent the P-4 and methylene blue, respectively. *F* represents absorption correction factor calculated by $F = 1 - 10^{-OD}$ (OD represents absorbance of the MB and P-4 at 442 nm). *S* represents the slope of absorbance plot of DPBF (416 nm) *vs.* irradiation time.

Photothermal effect of P-4 NPs was detected by IR thermal camera to observe temperature changes. PBS dispersion of P-4 NPs was exposed to the laser (660 nm, 0.8 W cm^{−2}) at different concentrations (0, 20, 40, 60 and 80 μg mL^{−1}) and different pH values (7.4 and 5.0) for 10 min.

For assessing photo-stability of P-4 NPs, DLS morphology and UV-vis-NIR spectra before and after four cycles of heating (660 nm, 0.8 W cm^{−2}, laser on: 5 min) and cooling (660 nm, 0.8 W cm^{−2}, laser off: 5 min) were documented.

To evaluate the photothermal conversion (PTC) efficiency, PBS dispersion of P-4 NPs was irradiated under 660 nm laser (0.8 W cm^{−2}, 10 min). When the temperature reached a plateau, the laser was turned off. During the procedure, the real-time temperature change was recorded by IR thermal camera per 20 s in 20 min. PBS of the same volume was also measured as control. The PTC efficiency (η) was calculated by the following equation:

$$\eta = \frac{hS(T_{\max} - T_{\text{sur}}) - Q_0}{I(1 - 10^{-A_{660}})} \quad (1)$$

where, *h* is the heat transfer coefficient; *S* is the surface

area of sample container; $T_{\max}$ is the steady-state maximum temperature; T_{surr} is the ambient room temperature; Q_0 is the energy input after laser irradiation by the same solvent without NPs in the same condition. hS and Q_0 were calculated by the following equations, respectively:

$$\tau_s = \frac{C_d m_d}{hS}, \quad (2)$$

$$Q_0 = hS(T_{\max} - T_{\text{surr}}), \quad (3)$$

where, τ_s is the sample-system time constant; C_d of deionized water is about $4.2 \text{ J g}^{-1} \text{ K}^{-1}$; m_d is the mass of the PBS dispersion.

Cell culture and incubation condition

HeLa cells were incubated at 37°C in Dulbecco's Modified Eagle's Medium (DMEM), including 10% inactivated FBS and 1% double resistant (penicillin and streptomycin) under 95% air and 5% CO_2 atmosphere humidified condition.

In vitro cell cytotoxicity

After incubation of the HeLa cells with fresh medium containing different concentrations of P-4 NPs (0, 4, 8, 12, and $16 \mu\text{g mL}^{-1}$) in 96 well plates for 24 h, one plate was irradiated with laser (660 nm , 0.8 W cm^{-2} , 8 min) and the other was still kept in dark. After 12 h, the MTT solution (5 mg mL^{-1} , $20 \mu\text{L}$) was added to per well for 4 h. Then DMSO ($150 \mu\text{L}$ per well) was added to dissolve the purple formazan. At last, the absorbance was detected *via* a microplate reader at 492 nm . Cell viability values were calculated by: cell viability (%) = (absorbance of experimental group/absorbance of control group) $\times 100\%$.

Apoptosis study by flow cytometry

After incubation of the HeLa cells with fresh medium containing different concentrations of P-4 NPs (0, 4, 10 and $16 \mu\text{g mL}^{-1}$ P-4 NPs), all the cells were irradiated by 660 nm laser (0.8 W cm^{-2} , 8 min). and stained with propidium iodide and annexin V-FITC. At last, all samples were detected by flow cytometer.

AM/PI staining

The P-4 NPs incubated HeLa cells in two groups (0 and $16 \mu\text{g mL}^{-1}$) were irradiated by 660 nm laser (0.8 W cm^{-2} , 10 min) and stained with AM/PI (AM: $2 \mu\text{mol L}^{-1}$, PI: $8 \mu\text{mol L}^{-1}$). Then fluorescence images were acquired *via* an inverted fluorescence microscope to detect the living and dead cells (AM excited at 465 nm , PI excited at

533 nm).

In vitro cellular uptake of P-4 NPs

After incubation of the HeLa cells with fresh medium containing $10 \mu\text{g mL}^{-1}$ P-4 NPs in PBS (pH 7.4 and 5.0) dispersion in dark for 24 h (called this method as A), the cells were incubated with 4% paraformaldehyde for 20 min and washed by PBS. After being stained with DAPI for 3 min and rinsed with PBS thrice, the two plates were refilled for with PBS (1 mL, pH 7.4 and 5.0, respectively). The fluorescence images were measured by a laser scanning up-conversion luminescence microscope equipped (excited at 633 nm).

Intracellular ROS assay and sub-cellular localization

HeLa cells were incubated as above method (A). For ROS assay, all the cells were operated in 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 25 min, paraformaldehyde (4%) for 20 min and DAPI for 3 min in dark. After being washed thrice with PBS (pH 7.4), the plate was refilled with PBS (1 mL, pH 7.4) and irradiated with 660 nm laser (0.8 W cm^{-2} , 8 min). For sub-cellular localization assay, cells were respectively incubated with Green Lyso-Tracker, Mito-Tracker and ER-Tracker liquid dyes. At last, cell images were recorded with the laser scanning up-conversion luminescence microscope equipped (DCF excited at 488 nm , P-4 NPs and Green Trackers excited at 633 nm).

In vivo fluorescence imaging

The nude mice were anaesthetized with oxygen flow with 2% isoflurane (2 L min^{-1}) during imaging. And the contrast imaging at different time (0, 2, 6, 12, 24 and 48 h) were acquired *via* the fluorescence imaging system (excited at 660 nm). Afterwards, the mice were sacrificed by cervical dislocation. At last, major organs (tumor, heart, liver, spleen, lung and kidneys) were collected and imaged on the same imaging equipment.

Pharmacokinetics

Six SD rats with specific-pathogen-free (SPF) (three males and three females) were fasted for 12 h, and given P-4 NPs *via* intravenous injection at a dose of 3 mg kg^{-1} . The venous blood sample ($500 \mu\text{L}$) was drawn from the orbit of rats at 0, 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 6, 8, 12 h after intravenous administration. Plasma was separated from the whole blood by centrifugation at $3,000 \times g$ for 10 min at 4°C and stored at -80°C until ultraviolet spectrophotometer analysis. And the pharmacokinetic parameters were calculated by non-compartmental method

using DAS (Drug and Statistics) 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The area under the concentration-time curve (AUC) from zero to the last measurable plasma concentration point (AUC_{0-t}), AUC from 0 to infinity ($AUC_{0-\infty}$), terminal elimination half-life ($t_{1/2}$), mean residence time (MRT), and clearance (CL) were calculated systematically.

In vivo antitumor assay

Subcutaneous cells injection (5×10^6 cells in 200 μ L PBS) under forelimb method was used to obtain the HeLa tumor-bearing nude mice models. All mice were carefully looked after and randomly separated into three groups ($n = 6$, saline+laser, P-4 only and P-4+laser) in ventilated cages when the tumor volumes reached about 130–150 mm^3 . And the antitumor assay was operated with 0.4 mg kg^{-1} dosage of P-4 NPs for 10 min laser illumination (660 nm, 0.8 W cm^{-2}) every two days. The body weights and tumor volumes ($V = 0.5ab^2$, a : the largest superficial diameter; b : the smallest superficial diameter) were measured *via* digital scale and caliper.

Histology sample preparation

After 18 days treatment, to obtain the tumors and major organs (heart, liver, spleen, lung and kidney), all HeLa tumor-bearing nude mice were killed for histological analysis *via* haematoxylin and eosin (H&E) staining. At

last, all samples were imaged with microscopy.

RESULTS

Synthesis and characterization

Scheme S1 shows the synthetic process for P-4. 4-Butoxybenzaldehyde reacted with dipyrromethane to give purple P-1. Bromination of P-1 in the presence of *N*-bromosuccinimide occurred to afford purple P-2. Then P-3 was obtained by the reaction between P-2 and zinc acetate. At last, P-3 reacted with *N,N*-diethyl-4-ethynyl aniline to attain deep green P-4 in a high yield of 82%. ^1H NMR, ^{13}C NMR and MALDI-TOF MS (Figs S1–S3) were used to characterize all intermediates and P-4.

With the addition of CF_3COOH (TFA), H^+ can bond to pH-responsive DBAP groups in P-4, resulting in a series of changes, including solution color, absorbance and emission spectra. As shown in Fig. 1a, P-4 can be protonated to form P-4 1H and P-4 2H *via* gradually adding TFA. During this process, light green P-4 solution turned into brownish green (P-4 1H) and brown (P-4 2H), exhibiting a sensitive pH-responsive performance (Fig. 1b). As shown in Fig. 1c, P-4 exhibits characteristic absorption peaks at 471 and 674 nm, then blue-shift of the absorbance can be observed along with TFA addition due to the gradual protonation. Moreover, in corresponding emission spectrum (Fig. 1d), the characteristic emission peak of P-4 at 697 nm gradually blue-shifts and weakens,

Figure 1 (a) Proposed protonation mechanism of P-4 triggered by H^+ . (b–d) Color, absorbance and emission changes of P-4 solution (in DCM) with TFA addition.

which is attributed to the sequential protonation process as well, resulting in more energy delivering to non-radiative transition and intersystem crossing. All these experiments prove the pH-responsive behavior of P-4.

A simple self-assembly method was used to prepare the hydrophilic P-4 NPs [26]. When compared with P-4, Fig. 2a displays that P-4 NPs can superbly disperse in PBS. And the TEM image shows that P-4 NPs possess well-defined spherical structure at both pH 7.4 and 5.0. In addition, the DLS measurement suggests the NPs present narrow size distribution with average sizes 46 ± 2 nm (pH 7.4) and 68 ± 2 nm (pH 5.0) (Fig. 2b and Fig. S4a), which will promote the enhanced permeability and retention (EPR) effect. Furthermore, P-4 NPs exhibit a NIR absorbance band at 693 nm ($\lambda_{\max}$), demonstrating slight red-

shift (8 nm) compared with P-4 (Fig. 2c). It is attributed to the intermolecular and intramolecular π - π stacking of P-4, making P-4 NPs as the desired candidate for cancer phototherapy. Moreover, excellent molar extinction coefficient (ϵ) of P-4 NPs at 693 nm was $2.05(\pm 0.2) \times 10^4$ (mol L^{-1}) $^{-1} \text{cm}^{-1}$ ($A = \epsilon cL$) (Fig. 2d, Fig. S4b), proving the excellent light-absorbing capacity of the NPs. Additionally, emission peaks of P-4 and P-4 NPs (pH 7.4) are 703 and 714 nm, respectively (Fig. S4c). Besides, the emission peak of P-4 NPs (pH 5.0) is weak and blue-shift compared with that at pH 7.4 (Fig. S4d), which is consistent with the result in organic phase. At last, the fluorescence quantum yield (ICG as reference) was calculated to be 67.4%, implying the bioimaging capability of P-4 NPs (pH 7.4) for cancer phototherapy.

Figure 2 (a) Photographs of P-4 and P-4 NPs. (b) TEM image of P-4 NPs (pH 7.4), inset: DLS size distribution. (c, d) Absorbance of P-4 and P-4 NPs at different concentrations. (e) Fluorescence spectra of SOSG mixed with P-4 NPs (pH 7.4). (f, g) Photothermal curves of P-4 NPs at different concentrations and pH. (h) Heating and cooling curves of P-4 NPs ($80 \mu\text{g mL}^{-1}$) for four cycles (660 nm , 0.8 W cm^{-2}). (i) Photothermal curve of P-4 NPs (0.5 mL , $80 \mu\text{g mL}^{-1}$) during laser on and off (660 nm , 0.8 W cm^{-2} , control: PBS).

pH-responsive performance

Singlet oxygen ($^1\text{O}_2$) generation ability was measured by DPBF probe with TFA acidity regulator [27]. The absorbance of DPBF mixed with P-4 at low pH (with 25 mmol L^{-1} TFA) declined more rapidly with an irradiation time dependence compared with the groups of DPBF only and DPBF mixed with P-4 in neutral condition (Fig. S5a–d), which indicates an acidity-dependent $^1\text{O}_2$ generation ability of P-4. And compared with methylene blue reference (MB, $\Phi\Delta = 0.52$), the 84.3% (at low pH with 25 mmol L^{-1} TFA) $^1\text{O}_2$ quantum yield of P-4 is also much higher than that of MB and most reported agents [28,29]. Furthermore, the $^1\text{O}_2$ generation ability of P-4 NPs was also detected *via* indicator of $1.5\text{ }\mu\text{g mL}^{-1}$ SOSG [30]. As displayed in Fig. 2e (pH 7.4), the fluorescence of SOSG shows a steady increase with 660 nm laser illumination (at 531 nm, mixed with P-4 NPs), and P-4 NPs at pH 5.0 (Fig. S4e) present a stronger $^1\text{O}_2$ generation ability compared with that at pH 7.4, confirming the acidity-activated $^1\text{O}_2$ generation of P-4 NPs. Encouraged by the NIR absorbance of P-4 NPs, PTC ability of P-4 NPs (pH 7.4) was recorded *via* laser illumination (0.8 W cm^{-2}) for 10 min at different dispersion concentrations. Fig. 2f displays that the respective temperature increase is about 3.8, 11.0, 13.4, 14.7, and 17.3°C (from 0 to $80\text{ }\mu\text{g mL}^{-1}$), presenting obvious concentration dependence and excellent photothermal effect of P-4 NPs. Meanwhile, the temperature increase (19.5°C) of P-4 NPs ($80\text{ }\mu\text{g mL}^{-1}$) at pH 5.0 was also studied, which was superior than that of 17.3°C (pH 7.4) under the same condition, exhibiting a pH-responsive photothermal effect of P-4 NPs (Fig. 2g). To verify the stability of P-4 NPs in water, the Zeta potential was measured as -18.72 mV (Fig. S4g). Besides, Fig. 2h confirms that the temperature promotion of P-4 NPs has no decline after four periodic laser illumination cycles (on and off: respective 5 min), and the absorbance spectrum and size measurements of P-4 NPs exhibit no obvious changes (Fig. S5e, f). All these results indicate the excellent photostability of P-4 NPs. At last, the PTC efficiency was analyzed to be 30.0% (pH 7.4, Fig. 2i and Fig. S4f), which was better than that of some traditional inorganic agents, such as $\text{Fe}_3\text{O}_4\text{@PDA-5}$ (13.1%) and Cu_9S_5 (25.7%) [31,32]. And the individually PDT and PTT effects as controls can refer to our group's previous work [33].

In vitro examinations

The dark toxicity and photo-toxicity of P-4 NPs to HeLa cells *in vitro* were studied *via* MTT assay. As demonstrated in Fig. 3a, P-4 NPs possess superb biocompat-

ibility even at $16\text{ }\mu\text{g mL}^{-1}$ in dark and excellent photo-toxicity with low half maximal inhibitory concentration (IC_{50}) of $10\text{ }\mu\text{g mL}^{-1}$ by illumination. Besides, the flow-cytometric experiment was further carried out to quantitatively test the apoptosis at different stages triggered by P-4 NPs. As exhibited in Fig. 3b, the HeLa cell apoptosis rate is 6.7%, 31.1%, 49.9% and 75.2%, respectively, with a dose-dependence. Based on the superb PTT/PDT effect of P-4 NPs in a cellular microenvironment, the PTT toxicity alone (at room temperature) was explored by quenching the ROS with *N*-acetyl-L-cysteine (NAC, cell viability: 0.61) and PDT toxicity alone by maintaining the temperature of media at 4°C (cell viability: 0.78). For the synergistic PTT/PDT group, the cell viability is only 0.51. However, for the NAC + ice (4°C) group, the cell viability is around 0.94 (Fig. S6a, 660 nm laser, 0.8 W cm^{-2} , 5 min, $\text{IC}_{50} = 10\text{ }\mu\text{g mL}^{-1}$). At last, calcein-AM/PI co-staining was performed with HeLa cells to further observe the live (green) and dead (red) cells after 660 nm laser illumination.

Fig. 3c proves more dead cells after incubation with P-4 NPs compared with the control group (without P-4 NPs, Fig. 3d). All these results demonstrate that the P-4 NPs display high photo-ablation ability. Cellular uptake assay of P-4 NPs was executed after the P-4 NPs incubation using fluorescent confocal microscope. Cells in pH 7.4 present more obvious fluorescence than that in pH 5.0 (Fig. 4a), which implies that fluorescent radiation decay is declined in acidic TME. In addition, based on the superb $^1\text{O}_2$ generation capacity of P-4 and P-4 NPs in solvent, the $^1\text{O}_2$ generation capability was also estimated *in vitro* using DCFH-DA (excited at 633 nm) as ROS probe. The bright green fluorescence in cytoplasm reveals that P-4 NPs display desired $^1\text{O}_2$ generation capability in cancer cells (Fig. 4b). Furthermore, the subcellular localization experiment of P-4 NPs in HeLa cells was carried out by Green subcellular trackers. Fig. 4c presents the clear orange yellow color in the lysosomes, due to the fluorescence overlaps of green (Lyso-Tracker) and red (P-4 NPs). And the Pearson's correlation coefficient (PCC) for P-4 NPs and Lyso-Tracker channel was calculated to be 0.81, which was much higher than that for P-4 NPs and Mito-Tracker channel (0.45). It indicates the NPs can be mainly pumped into lysosomes (pH 4.5–5.0) *via* vascular-type H^+ -ATPase [20], which will trigger the improved phototherapy under acidic TME.

In vivo examinations

Based on the superb properties of P-4 NPs *in vitro*, a series of *in-vivo* experiments were implemented. At first, the fluorescence imaging of P-4 NPs was observed with

Figure 3 (a, b) MTT and flow cytometry assays (660 nm , 8 min , 0.8 W cm^{-2}). (c, d) Fluorescence images ($200\times$) of calcein-AM/PI co-staining HeLa cells incubated without and with P-4 NPs (660 nm , 10 min , 0.8 W cm^{-2} , $16\text{ }\mu\text{g mL}^{-1}$).

HeLa tumor-bearing nude mice. As presented in Fig. 5a, after the intravenous injection, fluorescence intensity steadily increased to maximum at 12 h, then decreased because of the metabolism of P-4 NPs, which will guide the cancer phototherapy *in vivo* on time window. Besides, the fluorescence of tumor and organs (spleen, kidney, lung, heart and liver) indicates the tumor and liver exhibit stronger fluorescence than other organs, which can further confirm that most of the NPs accumulate at tumor site *via* EPR effect. Then the liver is a detoxifying organ, demonstrating the desired metabolizable property of P-4 NPs *in vivo*. Additionally, photothermal imaging at tumor site was performed at 12 h intravenous injection with 660 nm laser irradiation (0.8 W cm^{-2}). Fig. 5b proves that the temperature at tumor site gradually rose to 51.6°C ($\Delta T = 15.4^\circ\text{C}$), but little temperature increased ($\Delta T = 3.7^\circ\text{C}$) in the control group was observed within 8 min,

which also verifies the superb tumor targeted accumulation and photothermal imaging effect of P-4 NPs for cancer phototherapy *in vivo*. Moreover, the pharmacokinetics of P-4 NPs was studied through the sensitive and specific ultraviolet spectrophotometer after intravenous injection. The mean plasma concentration-time profiles of P-4 NPs are shown in Fig. S7a. And the major pharmacokinetic parameters are presented in Table S1, which were calculated by a non-compartmental model. The ratio of $\text{AUC}_{0-1}/\text{AUC}_{0-\infty}$ was more than 80%, suggesting the blood collection time point was suitable for the pharmacokinetic studies. The data shows that the P-4 NPs were eliminated quickly, with a $t_{1/2}$ of $1.203 \pm 0.038\text{ h}$. The MRT and CL of P-4 NPs was $0.848 \pm 0.0515\text{ h}$ and $0.176 \pm 0.0145\text{ L h}^{-1}\text{ kg}^{-1}$, respectively.

Finally, the amplifying cancer phototherapy of pH-responsive P-4 NPs was carried out by 18 HeLa tumor-

Figure 4 (a, b) Confocal fluorescence images of HeLa cells incubated with P-4 NPs ($10 \mu\text{g mL}^{-1}$) and DCFH-DA after laser illumination. (c) Sub-cellular localization of P-4 NPs at pH 7.4 in HeLa cells. Scale bar: $10 \mu\text{m}$; P-4 NPs and trackers were excited at 488 nm.

Figure 5 (a) Fluorescence images of tumor and selected organs in tumor-bearing mice after intravenous injection of P-4 NPs. (b) Photothermal images of tumor-bearing mice in the absence and presence of P-4 NPs intravenous injection ($80 \mu\text{g mL}^{-1}$, $100 \mu\text{L}$). (c) Visual photographs of mice from three groups after 18 days treatment.

bearing nude mice, which were randomly divided into three groups ($n = 6$): (i) saline with laser irradiation, (ii) P-4 NPs without laser irradiation, (iii) P-4 NPs with laser irradiation as treatment group. Compared with (i) and (ii), tumors in (iii) were totally ablated at the second treatment and all black scars fell off with no recurrence

(18 days, 660 nm, irradiation time: 8 min). Nonetheless, all the tumor volumes expanded to $\sim 3,500 \text{ mm}^3$ after 18 days in control groups (Fig. S6b). And Fig. 5c visually shows the therapeutic result for the three groups after 18 days, demonstrating the amplifying phototherapeutic effect and no dark toxicity of P-4 NPs *in vivo*. Further-

more, the H&E staining of tumors for different groups after sacrificing the mice verifies that the cells of two control groups are in good condition compared with the treatment group, further revealing no side effect and great cancer ablation ability of P-4 NPs (Fig. S6d). Besides, the normal body weight change (Fig. S6c) and fine H&E staining of organs (spleen, kidney, lung, heart and liver) (Fig. S7b) can also prove no side effects of P-4 NPs.

CONCLUSIONS

In conclusion, we have successfully synthesized a pH-responsive and lysosome targeting zinc(II) metalated porphyrin (P-4), which display high NIR absorbance, desired fluorescence quantum yield, excellent singlet oxygen generation ability and superb PTC efficiency. *In vitro* experiments also prove the strong stability, low dark toxicity and desired photo-toxicity of P-4 NPs. Moreover, P-4 NPs could trigger tumor photo-ablation *via* single laser illumination *in vivo*, achieving highly efficient cancer PDT/PTT. In future, the pH-responsive therapy system can also be extended to other phototherapy agent for targeted and efficient cancer therapy.

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Author contributions Dong X designed the project. Dong X, Si W and Yang Z guided the project. Liang P and Tang H performed the experiments. Gu R, Xue L, Chen D and Wang W performed some supplemental experiments. Dong X, Si W and Liang P analyzed the results and wrote the manuscript. All authors participated in general discussion of the paper.

Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information The synthesis of *N,N*-dibutyl-4-ethynylaniline and supporting results are available in the online version of the paper.

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一种用于增强光动力/光热协同肿瘤治疗的酸性刺激响应锌(II)金属卟啉化合物

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摘要 肿瘤酸性微环境是低氧条件下糖酵解引发的, 它能激发体系的酸性响应系统, 为肿瘤靶向光治疗建立一定的触发机制. 金属卟啉结构具有单态氧产率高、荧光成像能力强等优点, 在生物医学领域得到广泛研究. 本文设计合成了一种具有优异荧光量子产率(67.4%)、高单态氧量子产率(84.3%)和理想光热转换效率(30.0%)的酸性响应锌(II)金属卟啉(P-4), 研究了其酸性肿瘤微环境刺激响应光动力/光热治疗性能. 自组装的P-4纳米粒子可以特异性地靶向溶酶体亚细胞器位点, 实现二丁胺苯基(DBAP)的质子化过程且伴随高光毒性; 在660 nm单激光照射下, 肿瘤被完全消除且无副作用, 表明酸刺激响应的P-4纳米粒子可以为肿瘤协同治疗提供一种新途径.