Supplementary Information

Aqueous colloidal nanoplatelets for imaging and improved ALA-based photodynamic therapy of prostate cancer cells

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Materials and Methods

Materials: 4-Aminolevulinic acid hydrochloride (ALA) was obtained from Research Products International (USA). 2-Mercaptopropionic acid (2-MPA) and methanol (≥99.7 %) were purchased from Merck. RPMI 1640, trypsin EDTA, and penicillin-streptomycin solutions were obtained from Multicell, Wisent Inc. (QC, Canada). Fetal bovine serum (FBS) was purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Phosphate-buffered saline (PBS) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Biomatik Corp. (ON, Canada). 96-well plates were purchased from Nest Biotechnology Co., Ltd (Wuxi, China). Cadmium acetate dihydrate ($Cd(OAc)_2 \cdot 2H_2O$) (>98 %), oleic acid (OA) (90 %, technical grade), selenium (powder, 100 mesh, 99.99 %, trace metal basis), hexane (HPLC grade, ≥97.0 %), 1- octanethiol (≥98.5 %), oleylamine (OLA, 70%) , 1-octadecene (ODE, 90%), ethanol (absolute), n-hexane (≥97.0 %), cadmium nitrate tetrahydrate (>99.0 %), sulfur (powder, 99.98 %), sodium myristate (≥99 %) were purchased from Sigma-Aldrich. Only ultra-pure water (18.2 MΩ, Rephile Bioscience, and Technology, Shanghai, China) was used when needed.

Cadmium myristate synthesis: Synthesis of cadmium myristate was performed by following a previously reported method.[1] 1.23 g cadmium nitrate tetrahydrate and 3.13 g sodium myristate was dissolved in 40 mL and 250 mL methanol separately. Then these two solutions were mixed and stirred for 2 h at room temperature. The resulted cadmium myristate as white precipitate was filtered and washed with methanol. Finally, it was vacuum dried overnight.

4 monolayer (ML) CdSe NPLs synthesis: For the synthesis of 4 ML thick NPLs, we followed a protocol reported previously with slight modifications.[1] 150 mg cadmium myristate, 24 mg Se and 15 mL n-octadecene (ODE) were loaded into a 50 mL 3-neck flask and degassed under vacuum at 95 °C using a Schlenk line. Then, the temperature was raised to 240 °C under

a nitrogen atmosphere. When the color of the solution becomes deep orange (around 190 °C), 90 mg of $Cd(OAc)_2.2H_2O$ powder was added to the reaction mixture. After 8 min of growth time at 240 °C, 2 mL of oleic acid was injected, and the reaction was quenched with a water bath. The unreacted and undesired species were isolated by selective precipitation using hexane as solvent and ethanol as antisolvent. The final product was stored in hexane for further use.

Preparation of crown growth precursor: We prepared the crown growth precursor using a previously reported method.[2] 480 mg $Cd(OAc)_2.2H_2O$, 340 µL OA and 2 mL of ODE were loaded in a glass bottle with a magnetic stirrer, and the mixture was sonicated for 30 min. Then, it was placed on a hot plate at 200 °C and stirred for 7 min, followed by 3 min sonication in a sonicator. This 10 min step was repeated till a white homogeneous mixture was obtained.

Preparation of S-ODE solution: 240 mg sulfur (S) powder and 50 mL of ODE were loaded into a 100 mL flask and degassed for 10 min at room temperature under vacuum. Then, the temperature was raised to 200 °C under nitrogen. As the color of the solution becomes golden yellow, the reaction is let to cool down to room temperature.

CdSe/CdS core/crown NPLs synthesis: Synthesis of core/crown heterostructured NPLs were performed by following a previously established method with slight modifications.[2] 1 mL CdSe NPL in hexane, 5 mL ODE, and 100 µL oleic acid was loaded into a flask and at 80 °C for 30 min to remove hexane. Then the reaction atmosphere was switched to nitrogen, and a 600 µL crown growth solution with 900 µL S-ODE was injected into the NPL mixture. The temperature was raised to 240 °C and maintained till the desired crown growth was achieved determined by the absorption peak of the CdS crown layer. The reaction is quenched with a water bath. NPLs were isolated from the undesired species using selective precipitation, and the final product was stored in hexane.

Transfer of NPLs into water (2MPA-NPLs): Briefly, 2.5 ml aqueous 2MPA solution (0.15 M) was added to 2.5 mL NPLs in hexane, and a few drops of NaOH (1.5 M) were added. A

brief shaking transferred all NPLs to the aqueous phase. NPLs in water were filtered from a 0.45 nm sterile filter and stored at 4 °C.

ALA loading on 2MPA-NPLs: ALA was loaded electrostatically to 2MPA-NPLs. Electrostatic loading was confirmed by isothermal titration calorimetry: 2MPA-NPLs (1 mg/mL, 1 mL in water) were titrated with ALA (0.25 mL from 1 mg/mL in water), and strong bindings exotherms indicated strong and complete binding of zwitterionic ALA to anionic NPLs were observed. Therefore, ALA was added to NPLs, right before in vitro tests.

Characterizations: Hydrodynamic sizes and zeta potentials were measured using a Malvern Zetasizer Nano ZS. Cd-content of prepared NPLs was determined using an Agilent 7700XICP-MS (inductively coupled plasma-mass spectrometer). Samples were digested with a mixture of sulphuric and nitric acid. Absorbance spectra were obtained using a Shimadzu UV-VIS-NIR spectrophotometer in the 330-800 nm range. Photoluminescence (PL) spectra were recorded on an Agilent Cary Eclipse PL between 450-650 nm (λ_{exc}: 440 nm). For quantum yield measurements, samples were excited with a 405 nm laser (Cobalt Laser) in an integrating sphere, and the data was collected with an Ocean Optics S4000 spectrometer. Transmission electron microscopy (TEM) images were taken by JEOL TEM 2100F operated at 200 kV in the high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM).

Cell Culture: PCa cells LNCaP, PC3, DU145, and normal prostate epithelium PNT1A cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin-streptomycin and incubated in a 5 % CO2 humidified incubator at 37 °C.

Cytotoxicity assay: The cytotoxicity of free ALA, 2MPA-NPLs, and ALA/2MPA-NPLs were evaluated using MTT ((Thiazolyl blue tetrazolium bromide (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)) assay. Cells were seeded at a density of 1×10^4 cells/well into 96 well plates and incubated for 24 h. Then, the medium was replaced with a fresh medium,

containing free ALA or prepared NPLs. After 24 h incubation, cells were treated with 50 µL of MTT (5 mg/mL in PBS) solution and 150 µL fresh medium for 4 h. DMSO:Ethanol (1:1 v/v) was added to dissolve purple formazan crystals formed by the viable cells. The absorbance was read at 570 nm with a reference reading at 650 nm using Synergy, H1 (Biotek Instruments). Relative cell viability was calculated using the following formula:

Cell viability (%) = [$Absorbance_{sample}$ / $Absorbance_{control}$] x100

Untreated cells were used as control.

***In vitro* fluorescence imaging:** LNCaP, PC3, and DU145 were seeded at a density of 175,000 cells/well in 6 well plates and incubated for 24 h. Then, cells were treated with free ALA and ALA/2MPA-NPL at 0.44 mM ALA or 50 µg Cd /mL dose and incubated for 4 h and 24 h. The medium was removed, and cells were washed three times with PBS. Then, cells were fixed with 4% paraformaldehyde, washed with PBS and stained with DAPI (10 µg/mL). After 15 min incubation, cells were washed three times with PBS. Cells that were not treated with the test materials were used as control. Images were taken by Zeiss fluorescence microscope using separate filters for DAPI (λ_{exc} = 365 nm and λ_{em} = 417–477 nm), NPLs fluorescence (λ_{exc} = 494 nm and λ_{em} = 517 nm), and ALA (λ_{exc} = 591 nm and λ_{em} = 633 nm).

***In vitro* PDT:** Cells were seeded as described above and treated with free ALA, 2MPA-NPL, and ALA/2MPA-NPL between 0.044-0.44 mM ALA or 5-50 µg Cd /mL concentration range. After 4 h and 24 h incubation, the medium was replaced with fresh medium and cells were irradiated for 3 min with a 640 nm LED, Luzchem (10 mW/cm^2) from the top of the plate. MTT assay was used to determine the cell viability 24 h after irradiation. The same laser protocol was applied to cells that were not treated with the test materials to determine the safety of LED irradiation. Cells that were not treated with the test materials or with LED were used as a control.

Live/dead cell viability assay: Live/dead cell viability assay (The LIVE/ DEAD Viability/Cytotoxicity Kit, ThermoFisher Scientific) was used according to the manufacturer's instructions. Cells were seeded in 96 well plate as described above and treated with test materials at 0.44 mM or 50 µg Cd /mL dose. After 4 h incubation, the medium was replaced with fresh medium and cells were irradiated as described above. After 24 h incubation, cells were stained with calcein AM and ethidium homodimer-1. Images were obtained using a Zeiss fluorescence microscope (calcein: λ_{exc} = 494 nm and λ_{em} = 517 nm and ethidium homodimer-1: λ_{exc} = 528 nm and λ_{em} = 617 nm).

ROS generation: LNCaP, PC3, and DU145 cells were seeded and treated with free ALA and ALA/2MPA-NPL as described above. After 4 h incubation, the medium was replaced with fresh medium. Then, cells were irradiated for 3 min with a 640 nm LED (10 mW/cm^2). After 4 h incubation, each group was incubated with 10 µM DCFH2-DA for 40 min at 37 °C. Fluorescence intensity was read at λ_{exc}-λ_{em} =485-535 nm with a microplate reader (Synergy H1, Biotek Instruments).

Statistical analysis: Data's statistical significance was determined using Kruskall-Wallis one-way analysis of variance followed by Dunn's comparison test of the GraphPad Prism software package (GraphPad Software, Inc., CA, USA). P-value < 0.01 was considered for statistical significance. All data were reported as mean ± standard deviation (SD).

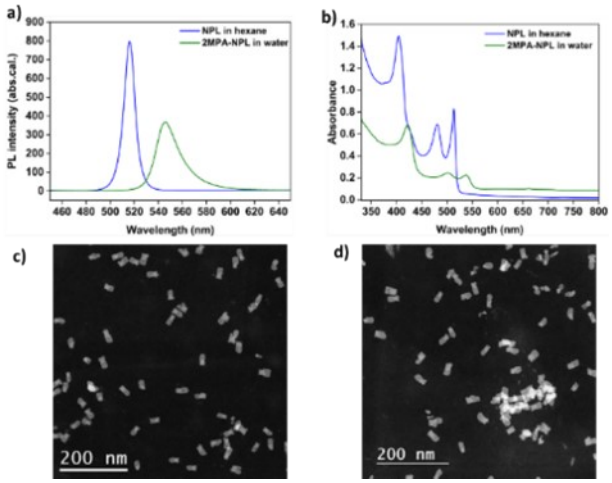

Fig.S1. a) The photoluminescence and b) absorbance spectra of NPLs in hexane and water, c) dark field transmission electron microscopy images of NPLs in hexane, and (d) in water.

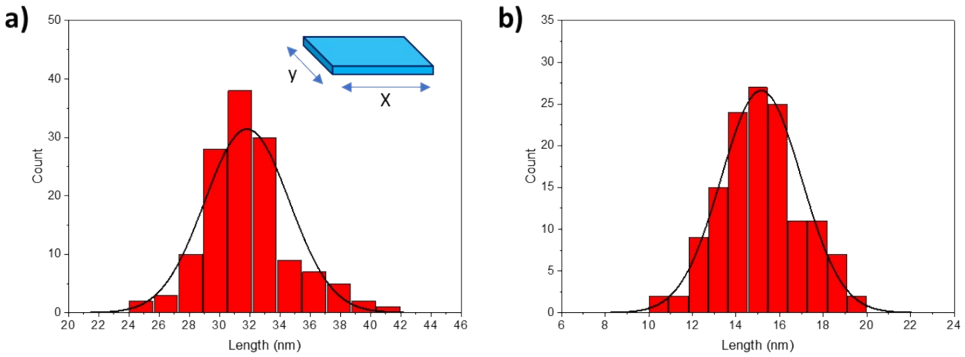

Fig. S2 Size histogram for a) x-, and b) y-dimensions of NPLs.

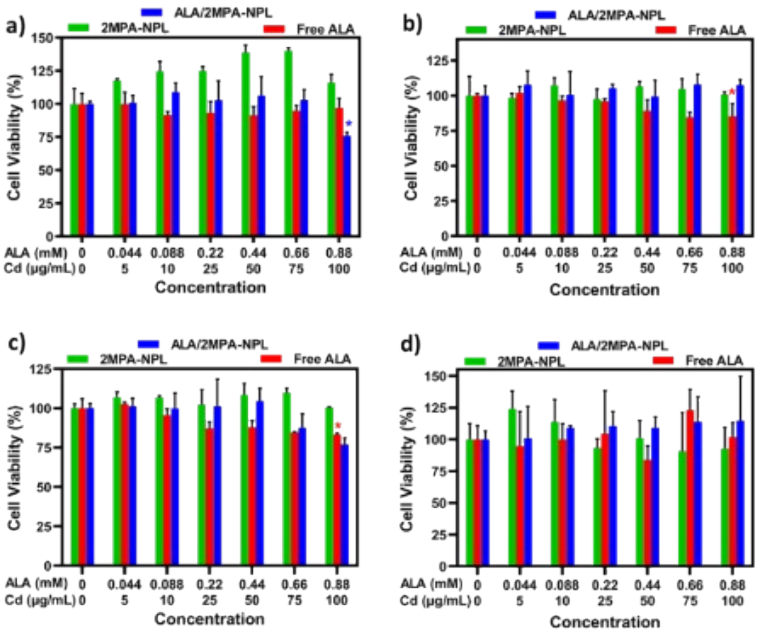

Fig. S3 Dose-dependent viability of a) LNCaP, b) PC3, c) DU145, and d) PNT1A cells treated with free ALA, 2MPA-NPL, and ALA/2MPA-NPL after 24 h incubation. Untreated cells were used as control. Data are expressed as mean ±SD (n=3).

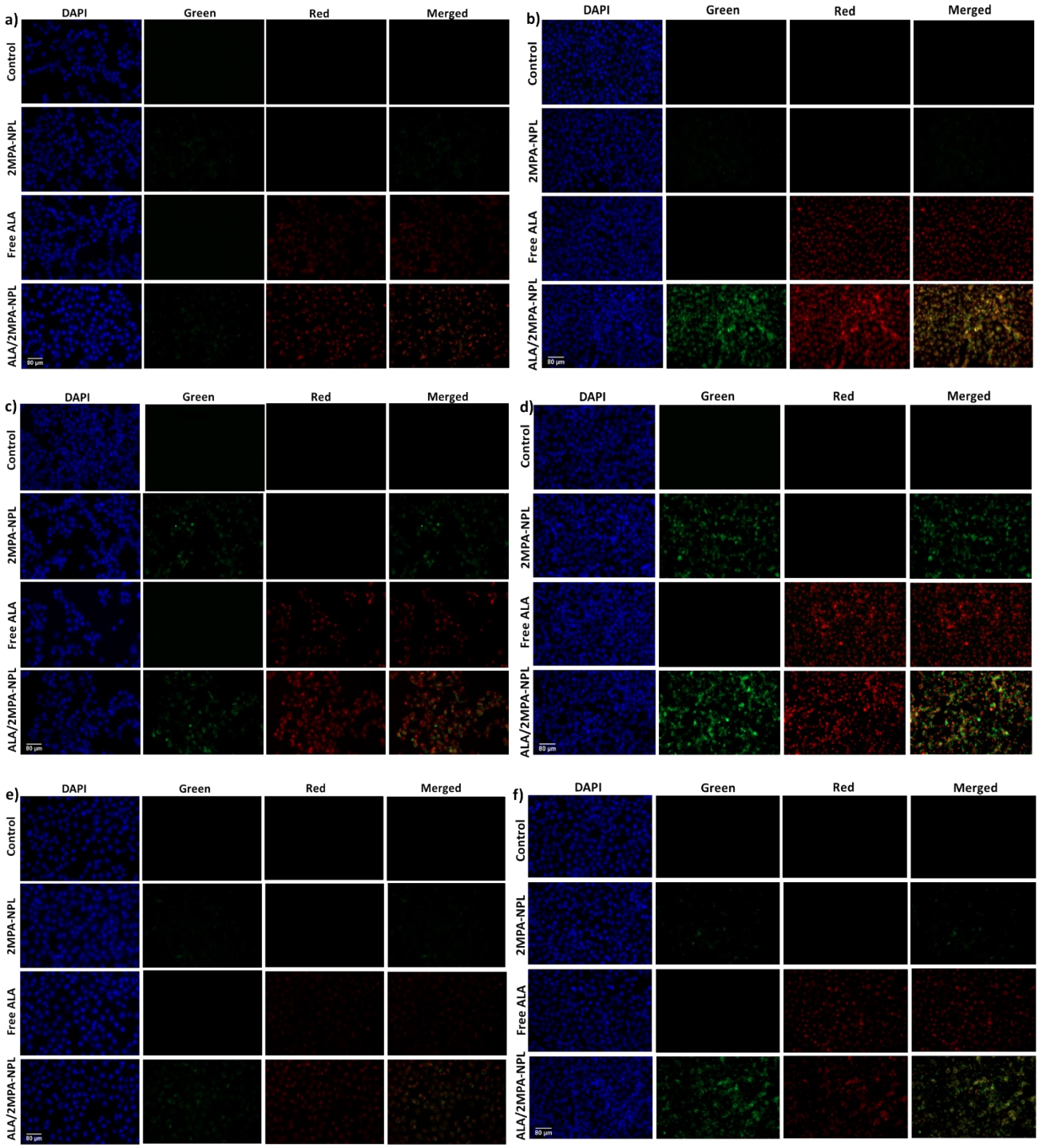

Fig. S4 Fluorescence microscopy images of 2MPA-NPL, free ALA, and ALA/2MPA-NPL treated a) and b) LNCaP, c) and d) PC3, and e) and f) DU145 cells after 4 h and 24 h incubation at 0.44 mM ALA or 50 µg Cd/mL.

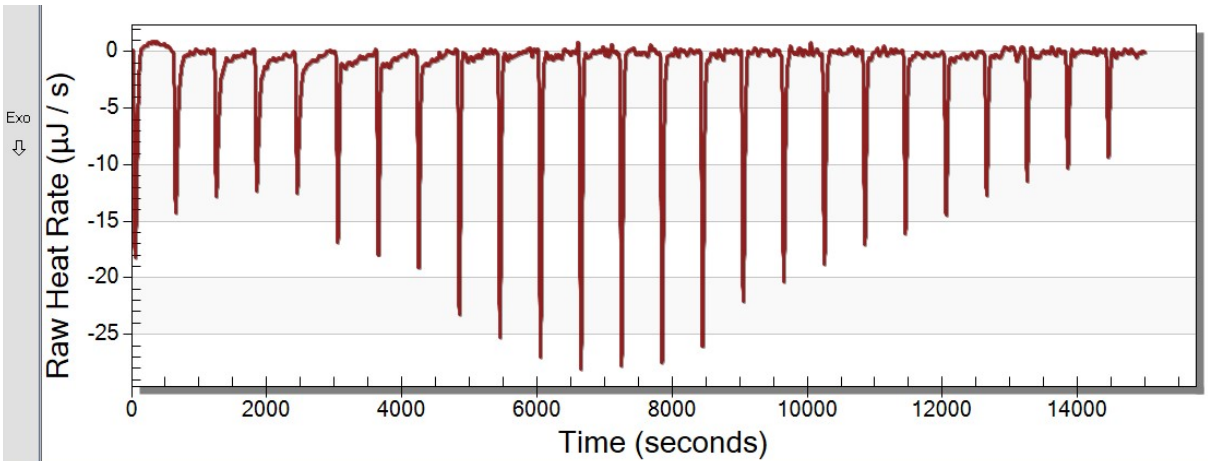

Fig. S5 Binding exotherms of ALA to 2MPA-NPL detected via isothermal titration calorimetry (ITC).

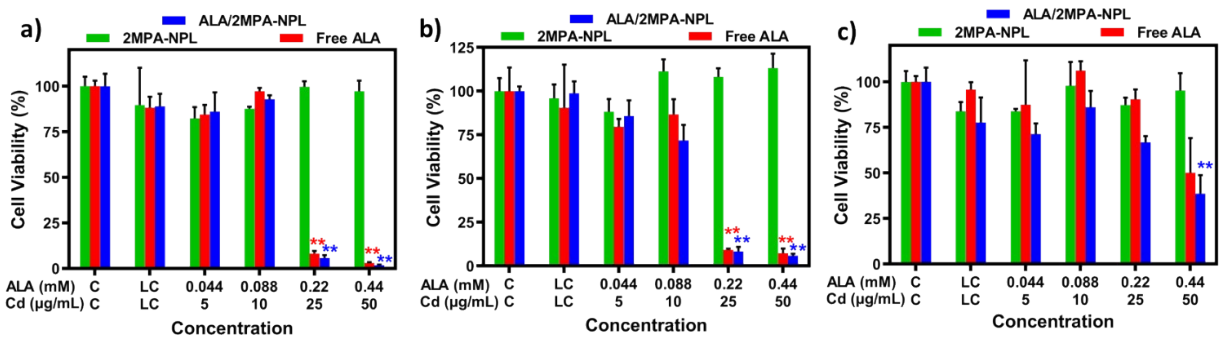

Fig. S6 Influence of free ALA, 2MPA-NPL, and ALA/2MPA-NPL on the viability of a) LNCaP, b) PC3, and c) DU145 cells as determined by MTT at different concentrations. LC: LED control, C: untreated cells. All other cells were irradiated with 640 nm LED at 10 mW/cm^2 for 3 min after 24 h incubation of the agents with the cells.

References

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2. M.D. Tessier, P. Spinicelli, D. Dupont, G. Patriarche, S. Ithurria and B. Dubertret, *Nano Lett.* 2014, *14* (1), 207-213.