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Supporting Information

A Two-Photon Ratiometric ESIPT Probe for Fast Detection and Bioimaging of Palladium Species

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Contents

- 1. Materials and general methods
- 2. Scheme caption and fluorescence spectra
- 3. OP and TP bioimaging experiment
- 4. NMR and ESI mass Data

1. Materials and general methods

Two-photon absorption cross sections were measured using the two-photoninduced fluorescence measurement technique. The two-photon absorption cross sections (δ) were determined by comparing their two-photon excitation fluorescence (TPEF) to that of fluorescein in different solvents, according to the following equation:

$$\delta = \delta_{ref} \bullet \frac{n_{ref}}{n} \bullet \frac{\Phi_{ref}}{\Phi} \bullet \frac{C_{ref}}{c} \bullet \frac{F}{F_{ref}}$$

In the equation, the subscript ref stands for the reference molecule. δ is the twophoton absorption cross-section value, n is the refractive index of the solution, Φ is the fluorescence quantum yield, c is the concentration of solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength.

The detection limit was calculated based on the fluorescence titration. The fluorescence emission spectrum of **1** was measured by ten times and the standard deviation of blank measurement was achieved. To gain the slope, the fluorescence intensity was plotted as a concentration of Pd⁰. So the detection limit of **1** was calculated with the following equation: Detection limit = $3\sigma/k$

Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus Pd⁰.

The solution of AlCl₃, NaCl, KCl, CaCl₂, BaCl₂, MgCl₂, ZnCl₂, CrCl₃, CdCl₂, CoCl₂, MnCl₂, LiClO₄, CuCl₂, FeCl₃, NiCl₂, Pb(NO₃)₂, HgCl₂, PtCl₂ and AuBr₃ were prepared in acetonitrile with a concentration of 10 mM, respectively. Pd(PPh₃)₄ was prepared in DMSO with a concentration of 10 mM, respectively. All the anion solutions were prepared from Na₂CO₃, NaBr, Na₂SO₄, NaNO₃, NaAcO, NaSCN, KI and NaF in distilled water, with a concentration of 10 mM, respectively.

2. Scheme caption and fluorescence spectra



Scheme S1. Preparation of probe 1 and its cleavage to form 2



Fig. S1. a) Fluorescence spectral change of **1** (10 μ M) upon addition of Pd(PPh₃)₄ (10 μ M) with micromolar NaBH₄ in water. $\lambda_{ex} = 380$ nm. Slit: 5.0 nm/5.0 nm; b) The time-dependent fluorescence change acquired in 20 min.



Fig. S2. a) Changes in fluorescence of 1 (10 μ M) upon addition of Pd⁰ (5 equiv.) with various mental ions (5 equiv.) Al³⁺, Na⁺, K⁺, Ca²⁺, Ba²⁺, Mg²⁺, Zn²⁺, Cr³⁺, Cd²⁺, Co²⁺, Mn²⁺, Li⁺, Cu²⁺, Fe³⁺, Ni²⁺, Pb²⁺, Hg²⁺, Pt²⁺ and Au³⁺; b) Fluorescence responses of 1 and 1-Pd⁰ (10 μ M) with various anions (5 equiv.): CO₃²⁻, Br⁻, SO₄²⁻, NO₃⁻, AcO₂⁻, SCN⁻, I⁻ and F⁻ in PBS buffer solution (pH = 7.4, containing 10% THF).



Fig. S3. The fluorescence intensity ratios (I_{552nm}/I_{470nm}) of 10 μ M 1 and 1-Pd⁰ as a function of pH in PBS buffer solution (containing 10% THF).



Fig. S4. Two-photon absorption cross section of **1** and **1**-Pd⁰ in PBS buffer solution (pH = 7.4, containing 10% THF).

3. OP and TP Bioimaging

3.1. Cell cytotoxicity

The cytotoxic effect of 1, 1-Pd⁰ and NaBH₄ was determined by an MTT assay following the manufacturer instruction (Sigma-Aldrich, MO). HeLa cells were initially propagated in a 25 cm² tissue culture flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 μ g/mL), and streptomycin (100 μ g/mL) in a CO₂ incubator. For cytotoxicity assay, cells were seeded into 96-well plates (approximately 104 cells per well), and various concentrations of NaBH₄ (0.01, 0.1, 1, 5, 10, 20, 50 µM), 1 and 1-Pd⁰ (5, 10, 20, 30, 50, 75 and 100 μ M) with 10 nM of NaBH₄ made in DMEM were added to the cells and incubated for 24 h. Solvent control samples (cells treated with DMSO, and THF) and cells treated with Pd^0 (100 μ M) alone were also included in parallel sets. Following incubation, the growth media was removed, and fresh DMEM containing MTT solution was added. The plate was incubated for 2-3 h at 37 °C. Subsequently, the supernatant was removed, the insoluble colored formazan product was solubilized in DMSO, and its absorbance was measured in a microtiter plate reader (Infinite M200, TECAN, Switzerland) with an excitation wavelength at 550 nm. The assay was performed in six sets for each concentration of 1 and 1-Pd⁰. Data analysis and calculation of standard deviation was performed with origin 8.0. For statistical analysis, a one way analysis of variance (ANOVA) was performed using Sigma plot.



Fig. S5. MTT assay to determine the cytotoxic effect of (a) **1** and $1-Pd^0$, (b) NaBH₄ on HeLa cells. Statistically significant values derived by ANOVA are indicated by bar marks.

3.2. Cell culture and tissue culture

HeLa cells were procured from the biomedical engineering center of Lanzhou University (Lanzhou, China). The cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 µg/mL), and streptomycin (100 µg/mL). Cells were maintained under a humidified atmosphere of 5% CO₂ and at 37 °C incubator as mentioned before. For cell imaging studies, cells were seeded into a confocal dish and incubated at 37 °C in a CO₂ incubator for one day. Then the cells were washed three times with PBS buffer (pH = 7.4) and divided into several groups. For group one cells were washed three times with phosphate buffered saline (pH = 7.4) and incubated with 10 μ M 1 in DMEM at 37 °C for 30 minutes in a CO₂ incubator and observed under Olympus FV1000 laser confocal microscope IX81. For group two cells incubated with 5 µM Pd^0 were again washed thrice with PBS (pH = 7.4), and then incubated in phosphate buffered saline with 10 nM NaBH₄ for 60 minutes. At last, the cells loaded with 10 μ M 1. For group three, cells incubated with 10 μ M Pd⁰ were again washed thrice with PBS (pH = 7.4), and then incubated in phosphate buffered saline with 10 nM NaBH₄ for 60 minutes. At last the cells loaded with 10 µM 1. Again, images were taken using Olympus FV1000 laser confocal microscope IX81. The cells were washed with PBS three times to remove free compound before analysis. Then, fluorescence microscopic images were acquired. Also the concentration based imaging detected as the previous procedure.

Tissue slices were prepared from Hela cancer cells. A total of 2×106 Hela cancer cells diluted in 100 µL of serum-free PBS medium were injected subcutaneously into the right flank of 6- to 8-week-old BALB/c-nude mice to inoculate tumors. After Hela cancer cells inoculation was for 15 days, mice were sacrificed. Tumors were transferred and embedded with O.C.T (Sakura Finetek, USA, Torrance, CA) for frozen sections. The tissues were cut into 700 µm- thick slices using a vibrating-blade microtome. Slices were incubated with Pd⁰ (50 µM) for 12 h and then phosphate buffered saline with 50 nM NaBH₄ for another 12 h at 4 °C. After washing with PBS

for three times, the probe (50 μ M) was incubated for 12 h at 4 °C, and the slices were mounted with 10% glycerol and sealed with nail varnish on a glass substrate.

3.3. Fluorescence imaging for cells

Two-photon fluorescence images of dye labeled cells were obtained by exciting the probes with a modelocked titanium-sapphire laser source (Mai Tai DeepSee, 80 MHz, 90 fs) set at wavelength 760 nm with Olympus FV1000 laser confocal microscope IX81 with 40 objective, numerical aperture (NA)=0.6, and ZOOM*2. The images signals at 425-500 and 525-600 nm range were collected by internal PMTs in 12 bit unsigned 1024*1024 pixels at 40 Hz scan speed.

One-photon fluorescence images of dye labeled cells were obtained with exciting wavelength at 380 nm with Olympus FV1000 laser confocal microscope IX81 with 40 objective, numerical aperture (NA)=0.6, and ZOOM*2. The images signals at 425-500 and 525-600 nm range were collected by internal PMTs in an 12 bit unsigned 1024*1024 pixels at 40 Hz scan speed.

3.4. Fluorescence imaging for tissue

Two-photon fluorescence images of dye labeled tissues were obtained by exciting the probes with a modelocked titanium-sapphire laser source (Mai Tai DeepSee, 80 MHz, 90 fs) set at wavelength 760 nm with Olympus FV1000 laser confocal microscope IX81 with 10 objective, numerical aperture (NA)=0.4, and ZOOM*1. The images signals at 425-500 and 525-600 nm range were collected by internal PMTs in 12 bit unsigned 1024*1024 pixels at 40 Hz scan speed.

One-photon fluorescence images of dye labeled tissues were obtained with exciting wavelength at 380 nm with Olympus FV1000 laser confocal microscope IX81 with 10 objective, numerical aperture (NA)=0.4, and ZOOM*1. The images signals at 425-500 and 525-600 nm range were collected by internal PMTs in an 12 bit unsigned 1024*1024 pixels at 40 Hz scan speed.

4. NMR and ESI mass Data



Fig. S6. ¹H NMR spectrum of **1** (DMSO-d₆).





Fig. S8. ESI mass spectrum of 1.



Fig. S9. High resolution mass spectrum (HRMS) of 1.



Fig. S10. ¹H NMR spectrum of 1-Pd (DMSO-d₆).



Fig. S11. ESI-mass spectrum of 1-Pd.



Fig. S12. ¹H NMR spectra of 1 and the product of 1-Pd⁰ in DMSO-d₆.