Electronic Supplementary Information for

Fluorescence turn-on H_2O_2 probe exhibits lysosome-localized fluorescence signals

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Materials and Experimental Details.

Commercially available chemicals were used as received. $FeSO_4 \cdot 7H_2O$ was purchased from Aldrich. Fresh Fe solutions (20 mM) were prepared in milli-Q grade water (18.2 MΩ·cm) before spectroscopic measurements. Fresh metal stock solutions (typically 0.1 or 0.01 M except for $CrCl_3 \cdot 6H_2O$, TCI) were prepared in milli-Q water using the corresponding chloride salts: $CuCl_2$ (99.999%, Aldrich), NaCl (≥99.5%, Aldrich), KCl (99.999%, Aldrich), $MgCl_2$ (99.99%, Aldrich), $CaCl_2$ (99.99%, Aldrich), $CrCl_3 \cdot 6H_2O$ (98%, Aldrich), $MnCl_2$ (99.99%, Aldrich), $FeCl_2$ (99.99%, Aldrich), $CoCl_2$ (99.9%, Aldrich), $NiCl_2$ (99.99%, Aldrich), $ZnCl_2$ (99.999%, Aldrich), $CdCl_2$ (99.999%, Aldrich), and $HgCl_2$ (99.999%, Aldrich). A K_2EDTA solution was prepared by dissolving EDTA dipotassium salt (≥99%, Sigma) in milli-Q water. PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), ≥99%) was purchased from Aldrich. A pH 7.0 buffer solution was prepared by dissolving PIPES (25 mM) in milli-Q water and adjusting the pH with standard KOH (45 wt %, Aldrich) and HCl (1N, Aldrich) solutions. The buffer solution was further treated with Chelex® 100 resin (BIO-RAD) to remove trace metal ions, filtered through a membrane (pore size = 0.45 μm). ZP1 was synthesized according to the method reported by Lippard and co-workers. ZP1 stock solution was dissolved in DMSO (Aldrich) to a 10 mM and stored frozen. The solution was thawed before spectroscopic measurements. Typically, 30 μL of the ZP1 stock solution and 30 μL of the Fe^{2+} solution were mixed in a small Eppendorf centrifuge tube and the mixed solution was delivered to 3 mL of pH 7.0 PIPES buffer to give a 10 μM solution. A 1 cm × 1 cm fluorimeter cell with a Teflon stopper (Hellma) was used for optical measurements. UV-vis absorption spectra were collected on a Varian Cary 50 spectrophotometer or an Agilent UV-vis 8453 spectrometer at 25 °C. Fluorescence spectra were obtained by using a Quanta Master 40 scanning spectrofluorimeter at 25 °C. The solutions were excited by using an excitation beam at 512 nm throughout fluorescence measurements. Iron titration experiments were performed by continuously adding 3 μL $FeSO_4$ stock solution (1 mM) to a 10 μM ZP1 in aqueous buffers (25 mM PIPES) at pH 7.0 per each titration step. H_2O_2 (30 wt %, Aldrich) was diluted in milli-Q water to 1000, 100 and 10 mM and a 30 μL portion of a H_2O_2 solution of an indicated concentration was added to a $ZP1Fe_2$ solution. The fluorescence quantum yield (Φ) was relatively determined using following standard equation:

$$\Phi_{sensor} = \Phi_{ref} \times \frac{I_{sensor}}{I_{ref}} \times \frac{A_{ref}}{A_{sensor}} \times \left(\frac{n_{sensor}}{n_{reference}}\right)^2$$

in which *A*, *I*, and *n* are absorbance at the excitation wavelength, integrated photoluminescence intensity (integration wavelength = 450 – 650 nm), and refractive index of solvent, respectively. Fluorescein as an aqueous 0.1 N NaOH solution was used for the external reference (Φ_{ref} = 0.79).

We assumed the refractive indices of the buffer and aqueous 0.1 N NaOH solutions to be identical. Solutions of $O_2^{\bullet-}$, OCl^-, *t*-BuO• and •OH were prepared by adopting the previous methods.[1,2] *t*-BuOOH, CAN, and DDQ were purchased from Aldrich and used without further purification. Photoirradiation (λ_{ex} > 500 nm) of an O_2-bubbled buffers (pH 7.0, 25 mM PIPES) containing methylene blue (1 μM) was performed to generate $^1O_2$. Nitric oxide (•NO) gas was obtained from Messer (Germany) and purified as follows: •NO gas was passed through two columns containing basic Ascarite II (NaOH fibrous silicate carrier) to remove higher nitrogen oxide impurities. Further purification by distillation was carried out by warming frozen •NO (as crystalline N_2O_2) from 78 K in a liquid N_2-cooled trap to 193 K through use of an acetone/dry-ice (–80 ºC) bath, and collection in a second liquid N_2-cooled evacuated trap. This secondary flask was again warmed to 193 K and the purified •NO (g) was collected in an evacuated Schlenk flask (50 mL) fitted with a septum. Standard KOH (45 wt %, Aldrich) and HCl (1N, Aldrich) solutions were used to prepare aqueous solutions of $ZP1Fe_2$ at pH 6.25, 6.71, 7.27, and 7.81. ^1H and ^{13}C NMR spectra were collected with a Bruker Ultrashield 400 plus NMR spectrometer and referenced to TMS. Electrospray ionization mass spectra were obtained by using a Thermo Electrons Co. Finnigan LCQ Advantage Max spectrometer. CW-EPR experiments were performed at room temperature using an X-band Bruker EMX-plus spectrometer equipped with a dual mode cavity (ER 4116DM). [Fe(DPAPhOH)] solution for the measurements was prepared by diluting a premixed solution of DPAPhOH (500 mM, DMSO) and $FeSO_4$ (500 mM, milli-Q water) in milli-Q water. 50 and 20 equiv H_2O_2 were added to the [Fe(DPAPhOH)] solution. Sample solutions (1 mM, ~300 μL) were loaded into EPR tubes (O.D. 4 mm). The experimental parameters for EPR spectra are as follows: Microwave frequency = 9.65 GHz, microwave power = 1 mW, modulation amplitude = 10 G, gain = 5×10^3, modulation frequency = 100 kHz, time constant = 40.96 ms, and conversion time = 85.00 ms.

Synthesis of DPAPhOH. To a 70 mL methanol containing salicylaldehyde (0.500 g, 4.09 mmol) and di-picolylamine (0.816 g, 4.09 mmol) was added two drops of glacial acetic acid. The reaction mixture was heated at 80 °C for 1 h, and cooled down to 0 °C by employing an ice bath. $NaBH_3CN$ (0.771 g, 12.27 mmol) was carefully delivered to the reaction mixture which was further stirred overnight at room temperature. The solution was poured onto a milli-Q water (70 mL), and the crude product was extracted with CH_2Cl_2 (150 mL × three times). The recovered organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated. Silica gel column purification was performed with CH_2Cl_2:CH_3OH = 19:1 to 9:1 (v/v) to afford brown liquid (0.673 g, 54%). ^1H NMR (400 MHz, *d*6-DMSO) □ 3.69 (s, 2H), 3.78 (s, 4H), 6.73 – 6.78 (br m, 2H), 7.09 (t, *J* = 4.0 Hz, 1H), 7.21 (d, *J* = 8.0 Hz, 1H), 7.27 (m, 2H), 7.44 (d, *J* = 8.0 Hz, 2H), 7.77 (td, *J* = 8.0, 2.4 Hz, 2H), 8.53

(d, J = 4.0 Hz, 2H), 10.53 (s, 1H). ^{13}C NMR (100 MHz, *d*6-DMSO) □: 54.48, 58.47, 115.74, 118.66, 122.36, 122.77, 123.44, 128.49, 130.12, 136.90, 148.78, 156.82, 158.35. MS (ESI, positive mode) m/z 306.1 ($[M+H]^+$).

Synthesis of [Fe(DPAPhOH)]. A 10 mL CH_3CN of 1 mM $Fe(ClO_4)_2$ was slowly layered onto a 10 mL CH_3OH of 1 mM DPAPhOH. 10 mL ether was carefully delivered to the mixture, which was left under anaerobic condition in a refrigerator. Colorless crystals were collected. A single crystal of $[Fe(DPAPhOH)(H_2O)(CH_3CN)](ClO_4)_2$ was picked from solutions by a nylon loop (Hampton Research Co.) on a hand made cooper plate mounted inside a liquid N_2 Dewar vessel at *ca*. −40 ºC and mounted on a goniometer head in a N_2 cryostream. Data collections were carried out on a Bruker SMART AXS diffractometer equipped with a monochromator in the Mo Kα (λ = 0.71073 Å) incident beam. The CCD data were integrated and scaled using the Bruker-SAINT software package, and the structure was solved and refined using SHEXTL V 6.12.[3] All non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were located in the calculated positions except H1m, H2m, and H3m, which were found from the Fourier difference map.

Cell Culture. HeLa and COS7 cells were grown in Dulbecco's modified Eagles's medium (DMEM), 10% fetal bovine serum (FBS), 50 μg mL^{-1} penicillin and 50 μg mL^{-1} streptomycin at 37 ^{o}C and 5% CO_2.

MTT Assays. Assays were carried out according to the manufacturer's protocol (EZ-Cytox, Dail Lab Service). Briefly, HeLa and COS7 cells were incubated with 2−20 μM $ZP1Fe_2$ or 1 μM staurosprin for 24 h, treated with MTT reagent for 1 h at 37 ^{o}C and MTT assay performed with the cells.

Fluorescence Microscopy. The cells were grown on a microscopic culture dish (diameter, 35 mm) with poly-L-lysine coating. Live cell imaging was obtained by Nikon A1R confocal microscope which was installed with a heated stage chamber (LCI, Korea) and supplied with 5% CO_2. The change of H_2O_2 level using $ZP1Fe_2$ was monitored with excitation at 488 nm and emission at 500−530 nm and the fluorescence of LysoTracker-Red was detected with excitation at 561 nm and emission at 575−625 nm. NIS-Elements Software was used for image analysis.

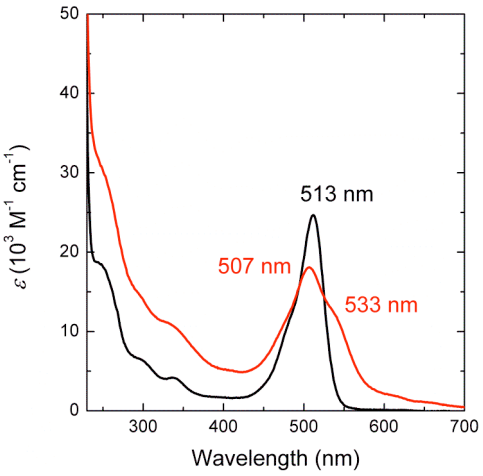

Figure S1. UV-vis absorption spectra of ZP1 (black line) and $ZP1Fe_2$ (red line). Condition: 10 μM in pH 7.0 buffer containing 25 mM PIPES and 25 °C.

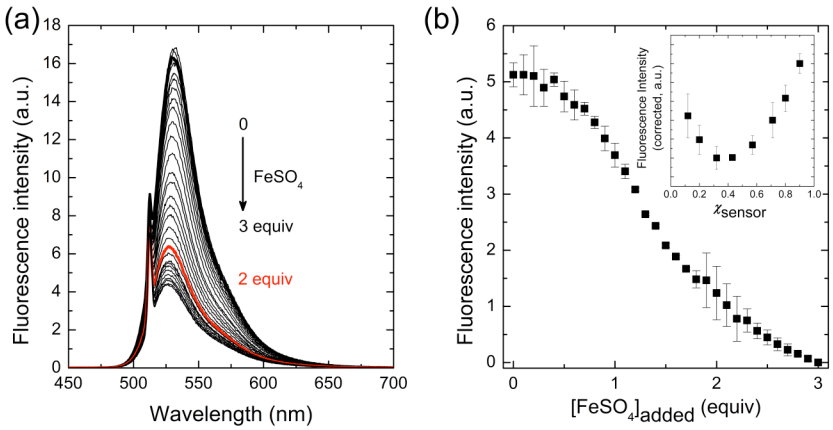

Figure S2. (a) Fluorescence change of 10 μM ZP1 (pH 7.0, 25 mM PIPES) with the continuous addition of $FeSO_4$ (0 – 3 equiv) and (b) corresponding titration isotherm. Titration experiments were performed in triplicate. Inset figure in (b) is Job's plot for the ZP1 and Fe binding ([ZP1] + [$FeSO_4$] = 10 μM).

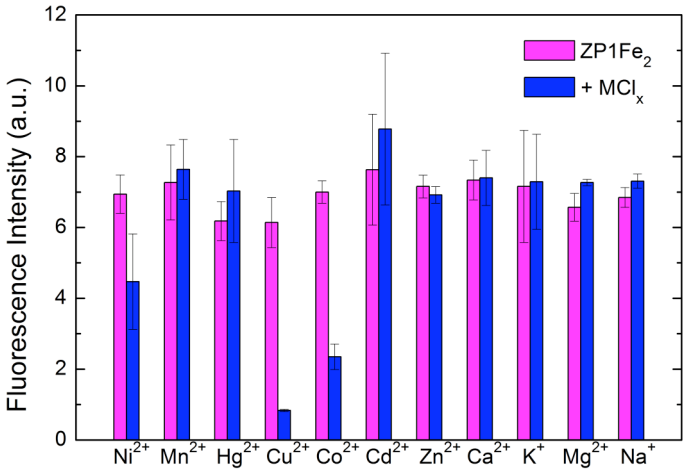

Figure S3. Fluorescence intensity of $ZP1Fe_2$ in the absence and presence of biologically important metal ions: 100 equiv Na^+, K^+, Ca^{2+}, and Mg^{2+}; 2 equiv Ni^{2+}, Mn^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+}. Chloride salts were introduced to 10 μM $ZP1Fe_2$ solution in pH 7.0 PIPES buffer (25 mM).

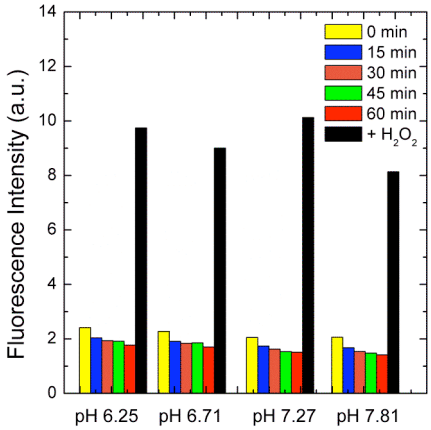

Figure S4. Stability of fluorescence of $ZP1Fe_2$ at various pHs. Fluorescence intensities of 10 μM $ZP1Fe_2$ in milli-Q water were recorded at indicated pHs for 1 h. H_2O_2 was delivered to the $ZP1Fe_2$ solution after 1 h.

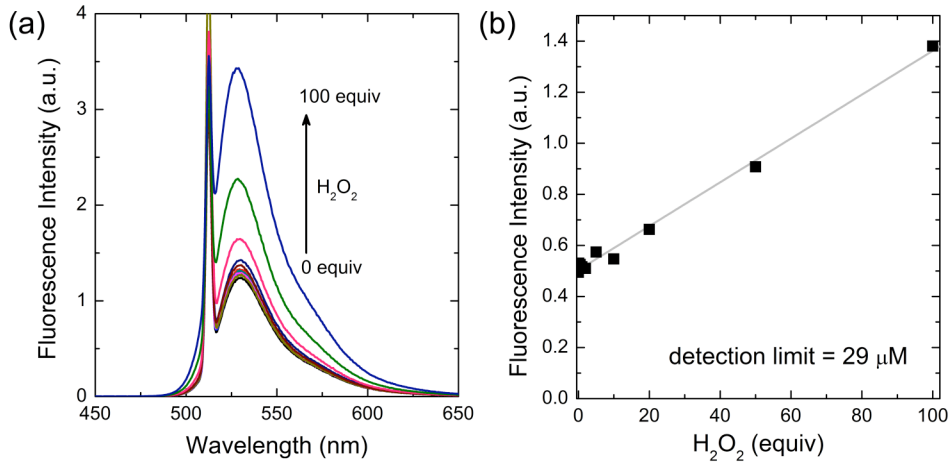

Figure S5. Determination of a limit of fluorescent detection of H_2O_2. PIPES buffer solutions containing 10 μM $ZP1Fe_2$ were incubated for 30 min by varying concentration of H_2O_2 (0 – 100 equiv).

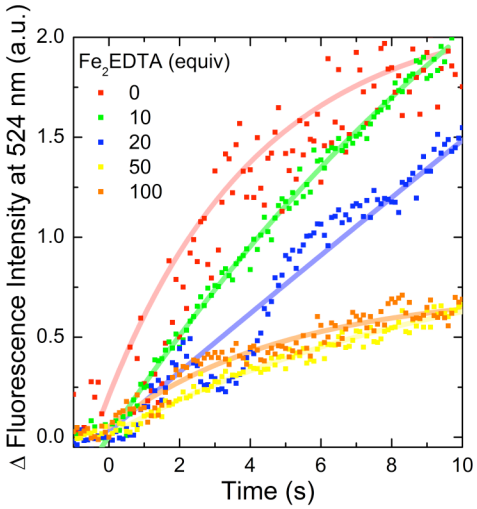

Figure S6. Increase in the fluorescence intensity of pH 7.0 aqueous buffers (25 mM PIPES) containing 10 μM $ZP1Fe_2$, 1.5 mM H_2O_2, and different concentrations of $FeSO_4$ and K_2EDTA (2:1; 0 – 100 equiv of K_2EDTA relative to $ZP1Fe_2$). Rates for fluorescence intensity increase at 524 nm are inversely proportional to the concentration of •OH. The fluorescence quenching at the high concentrations is due to the paramagnetic effect of Fe ions. The results imply that •OH exerts a negligible effect on the fluorescence turn-on.

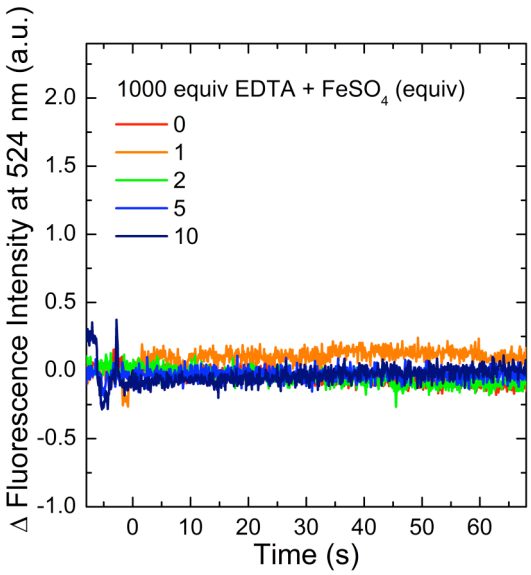

Figure S7. Increase in the fluorescence intensity of pH 7.0 aqueous buffers (25 mM PIPES) containing 10 μM ZP1, 1.5 mM H_2O_2, and different concentrations of $FeSO_4$ and K_2EDTA (1:1000; 0 – 10 equiv of Fe ions relative to ZP1).

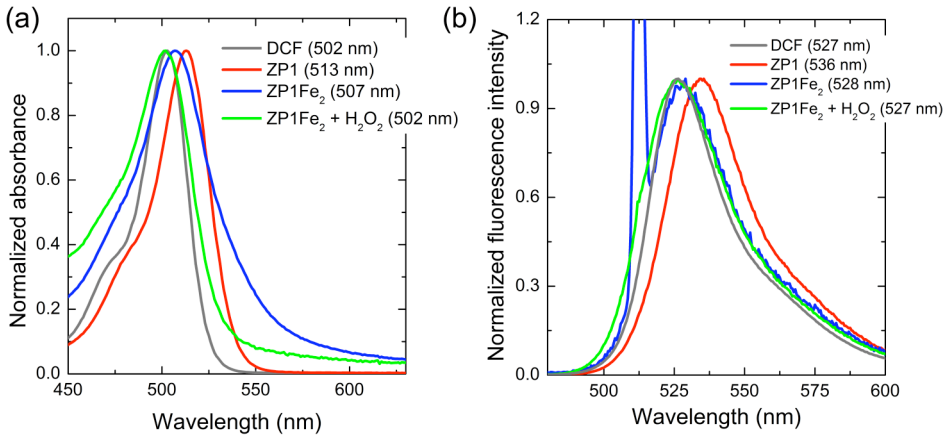

Figure S8. Normalized UV-vis absorption (a) and fluorescence (b) spectra of pH 7.0 aqueous buffer solutions (25 mM PIPES) of 2′,7′-dichlorofluorescein (DCF), ZP1, $ZP1Fe_2$, and the reaction mixture of $ZP1Fe_2$ and H_2O_2 (100 equiv relative to $ZP1Fe_2$). The peak wavelength of each species is indicated in the legend.

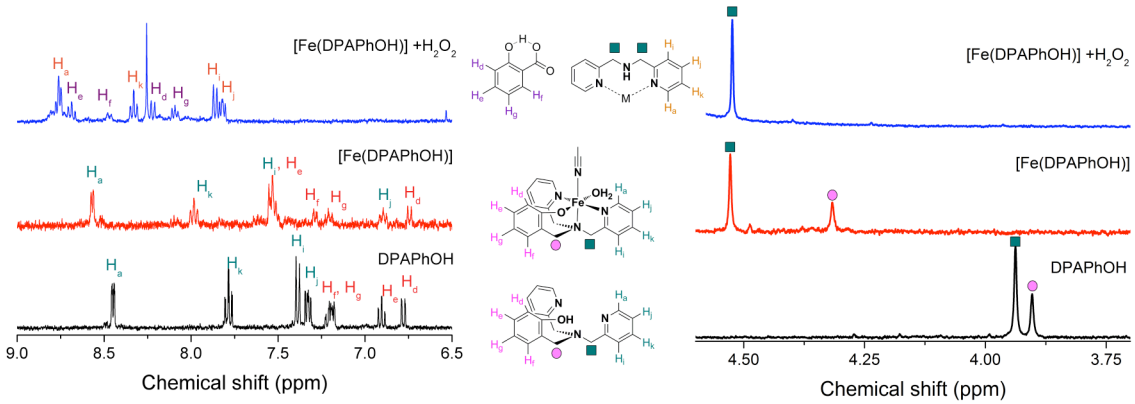

Figure S9. ^{1}H NMR spectra (400 MHz, 2.5 vol % *d*6-DMSO in D_2O) of 1 mM DPAPhOH, 1 mM [Fe(DPAPhOH)], and oxidation products of [Fe(DPAPhOH)] and H_2O_2 (50 equiv). The assignment of each peak is indicated in the middle structures.

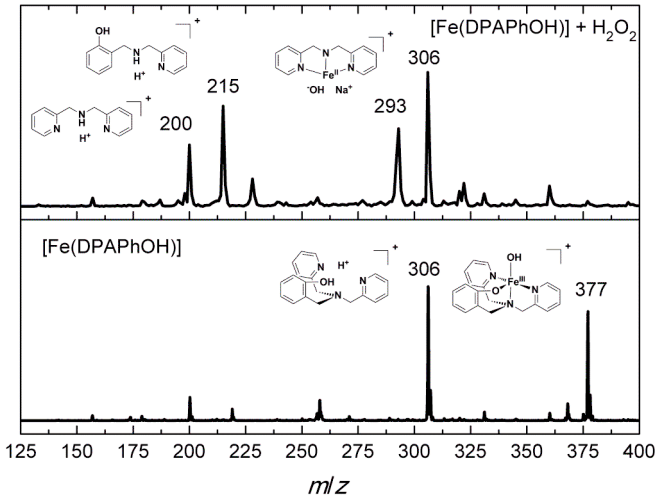

Figure S10. Positive ESI MS spectra of [Fe(DPAPhOH)] before and after reaction with H_2O_2. The dissociation counter part (i.e., salicylic acid (Mw = 138)) was not detected due to its low molecular weight.

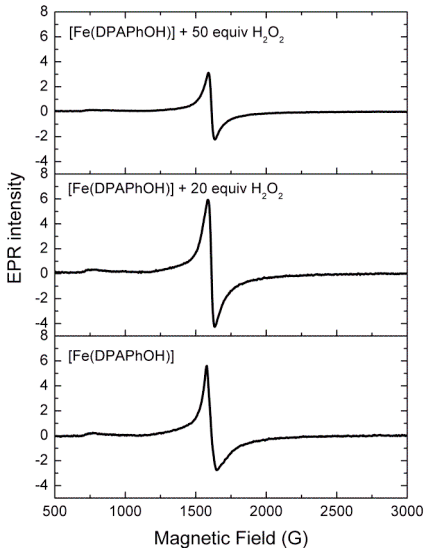

Figure S11. X-band CW EPR spectra of [Fe(DPAPhOH)] (1 mM in milli-Q water) before (g = 4.30) and after (g = 4.30) reaction with H_2O_2.

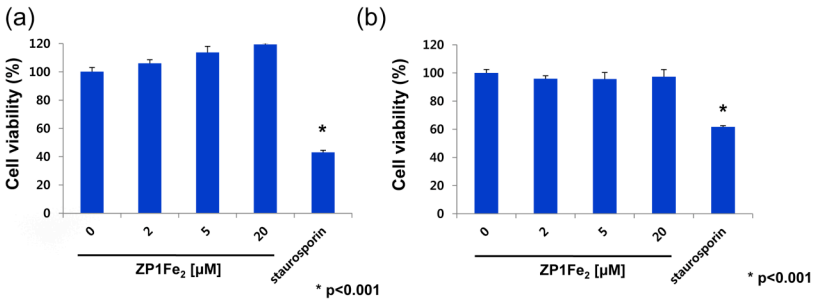

Figure S12. Cell viability data of HeLa cells (a) and COS7 (b) cells incubated with indicated concentrations of $ZP1Fe_2$ for 24 h.

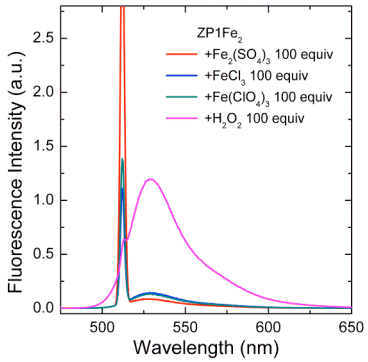

Figure S13. Fluorescence spectra of $ZP1Fe_2$ (10 μM) in the presence of $Fe_2(SO_4)_3$, $FeCl_3$, $Fe(ClO_4)_3$, and H_2O_2.

Table S1. Crystal data and structure refinements for [Fe(DPAPhOH)(H_2O)(CH_3CN)](ClO_4)$_2$.

	[Fe(DPAPhOH)(H_2O)(CH_3CN)](ClO_4)$_2$
Empirical formula	$C_{21}H_{24}Cl_2FeN_4O_{10}$
Formula weight	619.19
Temperature (K)	170(2)
Wavelength (Å)	0.71073
Crystal system/space group	Monoclinic, *Cc*
Unit cell dimensions	
a (Å)	11.3781(2)
b (Å)	18.5161(3)
c (Å)	12.4123(2)
α (°)	90
β (°)	91.2170(10)
γ (°)	90
Volume (Å^3)	2614.41(8)
Z	4
Calculated density (g/cm^{-3})	1.573
Absorption coefficient (mm^{-1})	0.842
Reflections collected	21950
Independent reflections [*R*(int)]	5695 [0.0270]
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	5695/2/ 356
Goodness-of-fit on F^2	0.913
Flack parameter	−0.001(11)
Final *R* indices [$I > 2\text{sigma}(I)$]	R_1 = 0.0304, wR_2 = 0.0802
R indices (all data)	R_1 = 0.0344, wR_2 = 0.0838

Table S2. Selected bond distances (Å) and angles (º) for [Fe(DPAPhOH)(H_2O)(CH_3CN)](ClO_4)$_2$.

Bond Distances (Å)	
Fe1-O1	2.1870(18)
Fe1-O2	2.123(2)
Fe1-N1	2.194(2)
Fe1-N2	2.154(2)
Fe1-N3	2.144(2)
Fe1-N4	2.090(2)
Bond Angles (°)	
N1- Fe1-N2	77.83(7)
N1- Fe1-N3	78.14(8)
N1- Fe1-N4	176.03(8)
N1- Fe1-O1	86.69(7)
N1- Fe1-O2	91.93(8)
N2- Fe1-N3	155.96(8)
N2- Fe1-N4	102.66(8)
N2- Fe1-O1	88.64(7)
N2- Fe1-O2	89.30(9)
N3- Fe1-N4	101.34(8)
N3- Fe1-O1	90.32(7)
N3- Fe1-O2	91.17(9)
N4- Fe1-O1	89.39(7)
N4- Fe1-O2	92.01(9)
O1-Fe1-O2	177.72(9)

References

1. Evan W. Miller, Aaron E. Albers, Arnd Pralle, Ehud Y. Isacoff, and Christopher J. Chang, *J. Am. Chem. Soc.*, **2005**, *127*, 16652–16659.
2. Yonggang Chen, Weihua Guo, Zhiqiang Ye, Guilan Wang, and Jingli Yuan, *Chem. Commun*. **2011**, *47*, 6266–6268.
3. G. M. Sheldrick, *SHELXTL/PC Version 6.12 for Windows XP*, Bruker AXS Inc., Madison, WI, 2001.