

Supporting Information

NHC-Modulated Fe(II) Complex for TADF-Assisted Multiphoton Lifetime Imaging: Concurrent Apoptosis Monitoring and Quantification

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Experimental methods

1.1 Materials and apparatus

All chemicals and solvents were dried and purified by usual methods. The synthetic routes for the compounds and their derivatives were illustrated in Supporting information. Mass spectra were performed on a Micromass GCT-MS spectrometer (UK). ^1H -NMR and ^{13}C -NMR of nuclear magnetic resonance were measured by Bruker Avance nuclear magnetic resonance apparatus. The infrared tester was a NEXUS 870 (Nicolet) type infrared spectrometer (KBr tablet, $4000\text{--}400\text{cm}^{-1}$). The determination of elemental analysis was performed by the Perking-Elmer 240B automatic elemental analyzer. UV-vis absorption spectra were recorded on a UV-265 spectrophotometer. Fluorescence measurements were carried out on a Hitachi F-7000 fluorescence spectrophotometer.

1.2. X-ray crystallography

Single-crystal X-ray diffraction measurements were carried out on a Bruker Smart 1000 CCD diffractometer equipped with a graphite crystal monochromator situated in the incident beam for data collection at room temperature. The determination of unit cell parameters and data collections were performed with Mo- K_α radiation ($\lambda=0.71073\text{ \AA}$). Unit cell dimensions were obtained with least-squares refinements, and all structures were solved by direct methods using SHELXL-97 program package. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were added theoretically and riding on the concerned atoms. The final refinement was performed by full-matrix least-squares methods with anisotropic thermal parameters for non-hydrogen atoms on F^2 .

1.3 Open-aperture Z-scan measurements

Z-scan test for the excitation light titanium sapphire laser, 680-1080 nm laser, by stepping machine to make samples in the focus of the lens ($z=0$) before and after the move, by test can directly find incident power and output power, and further get the test samples of the transmittance $T(z)$, normalized, $T(z) \sim z$ curve is the opening Z scan curve, the incident light focus $Z = 0$, there is an obvious valley, based on the gaussian pulse of time and space, the energy normalized transmittance $T(z)$ by the following equation:

$$T(z)=1-\frac{(q_0)^m}{(m+1)^{3/2}\left(1+\frac{z^2}{z_0^2}\right)^m}$$

Where $q_0 = \beta I_0 L_{\text{eff}}$ and $(m+1)$ is the number of photons absorbed.

In the case of two-photon absorption, this formula can be developed into:

$$T(z)=1-\frac{\beta I_0 L_{\text{eff}}}{2\sqrt{2}\left(1+\frac{z^2}{z_0^2}\right)}, \quad \beta = \frac{\sigma_2 N_A c \times 10^{-3}}{h\nu}, \quad L_{\text{eff}} = [1 - \exp(-a_0 L)] / a_0$$

In the case of three-photon absorption, this formula can be developed into:

$$T(z)=1-\frac{\gamma I_0 L_{\text{eff}}}{2\left(1+\frac{z^2}{z_0^2}\right)^2}, \quad \gamma = \frac{\sigma_3 N_A c \times 10^{-3}}{h\nu}, \quad L_{\text{eff}} = [1 - \exp(-2a_0 L)] / 2a_0$$

β is the two-photon absorption coefficient and γ is the three-photon absorption coefficient. σ_2/σ_3 is the two/ three-photon absorption cross-section. where N_A is the Avogadro constant, c is the concentration of the compound in solution, h is the Planck constant, and ν is the frequency of the incident laser beam. where a_0 is the absorption coefficient and L is a factor that depends on the actual sample length and the Rayleigh range.

1.4 Two-photon excitation fluorescence (2PEF) measurements

The 2PA coefficient (β) and the molecular 2PA cross-sections (σ) are obtained by an open-aperture Z-scan technique using a femtosecond laser pulse (680-1050 nm, 80 MHz). The pulse length is 140 fs, and the average laser power is 36 mW. The sample in DMSO (its thickness is 1 mm) is put in the light path, and all measurements are carried out at room temperature. The filled squares represent the experimental data, and the solid line is the theoretical curve modified from the following equations:

$$\sigma_{2s} = \frac{c_R \times n_s \times f_s \times Q_R}{c_s \times n_R \times f_R \times Q_S} \times \sigma_{2R}$$

where the subscripts “s” and “R” represent sample and reference (here, Rhodamine 6G in ethanol solution at a concentration of 1.0×10^{-3} mol/L was used as reference), respectively. f is the overall fluorescence collection efficiency intensity of the fluorescence signal collected by the fiber spectra meter. Q , n and c are the quantum yield of the fluorescence, the refractive index of solvent, and the concentration of the solution, respectively.

1.5 Three-photon excitation fluorescence (3PEF) measurements

All the samples are contained in 1cm-optical length quartz liquid cell and Rhodamin 6G as the standard sample for intensity comparison. Spectrometer: Ocean Optics QE65 Pro (300-2500nm). Laser: Coherent Astrella+TOPAS Prime (1200-2600) nm, 1 kHz, 120 fs. All the results provided here are the 10 times average results.

Theory:

$$\sigma_{3s} = \frac{c_R \times n_s \times f_s \times Q_R}{c_s \times n_R \times f_R \times Q_S} \times \sigma_{3R}$$

where the subscripts “s” and “R” represent sample and reference (here, Rhodamine 6G in ethanol solution at a concentration of 1.0×10^{-3} mol/L was used as reference), respectively. f is the overall fluorescence collection efficiency intensity of the fluorescence signal collected by the fiber spectra meter. Q , n and c are the quantum yield of the fluorescence, the refractive index of solvent, and the concentration of the solution, respectively.

1.6 Femtosecond Transient Absorption Spectroscopy

Femtosecond laser pulses were generated by a Ti:Sapphire amplifier operated at a 1 kHz repetition rate (Coherent, Astrella). The 800 nm fundamental passes through a beamsplitter, where most of the beam is sent into an optical parametric amplifier (Ultrafast Systems, Apollo-T) to generate 385 nm (200 μ w) pulses for the pump. S5 The rest of the fundamental is focused into a VIS continuum generation crystal (Ultrafast, Helios), to generate the white-light continuum. The pump pulse and probe were sent into an optical bench (Ultrafast, Helios). The time delay was adjusted by changing the path length of the probe. An optical chopper was used to block every other pump pulse. The signal was collected into a CCD camera after dispersion by a grating spectrograph (Ultrafast, Helios).

Kinetic traces were fitted to the convolution of the instrument response and a sum of exponential decays. Time zero was allowed to vary with wavelength to account for the chirp of the probe.

1.7 Cell Co-localization Assays

The subcellular localization experiments of $[\text{Fe}(\text{imp})_2]^{2+}$ are conducted with cell membrane (DiO)/mitochondria (Mito-Tracker Green)/nucleus (Hoechst 33342) commercial dyes.

Cell membrane co-localization: Hep G2 cells are cultured with $[\text{Fe}(\text{imp})_2]^{2+}$ (10 μM) for 10 min and then washed with PBS for two times. Then the cells are further treated with 2 μM DiO (Ex/Em = 484nm/501 nm) for 10 min, finally, washed with PBS for three time.

Mitochondria co-localization: Hep G2 cells are cultured with $[\text{Fe}(\text{imp})_2]^{2+}$ (10 μM) for 10 min and then washed with PBS for two times. Then the cells are further treated with 2 μM Mito-Tracker Green (Ex/Em = 490 nm/516 nm) for 10 min, finally, washed with PBS for three time.

Nucleus co-localization: Hep G2 cells are cultured with $[\text{Fe}(\text{imp})_2]^{2+}$ (10 μM) for 10 min and then washed with PBS for two times. Then the cells are further treated with 10 μM Hoechst 33342 (Ex/Em = 420 nm/460 nm) for 10 min, finally, washed with PBS for three time.

1.8 H_2O_2 induced apoptosis model

Hep G2 cells are incubated with 1-20 mM H_2O_2 for 12 h and then washed with PBS for two times. Then the cells are further treated with 10 μM $[\text{Fe}(\text{imp})_2]^{2+}$ (Ex/Em = 800 nm/500-650 nm) for 10 min, finally, gently wash with PBS for two time.

1.9 Glucose-induced cell death

Hep G2 cells are incubated with 0-60 mM Glucose for 0.5 h and then treated with 10 μM $[\text{Fe}(\text{imp})_2]^{2+}$ (Ex/Em = 800 nm/500-650 nm) for 10 min, finally, gently wash with PBS for two time.

1.10 Hg^{2+} induced death of heavy metals and LPS-induced pyroptosis

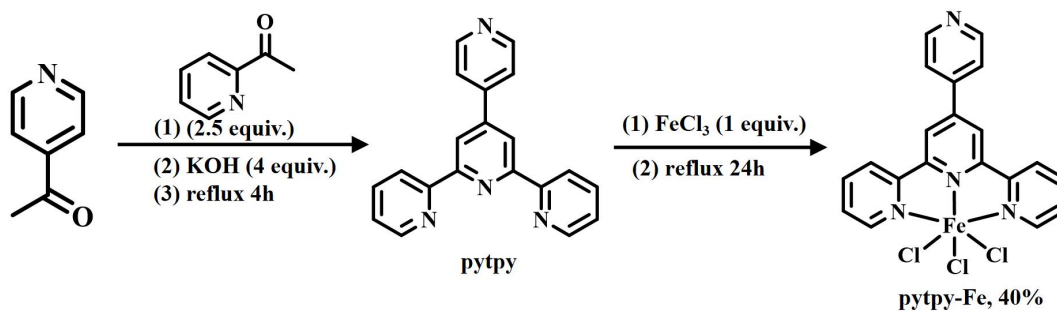
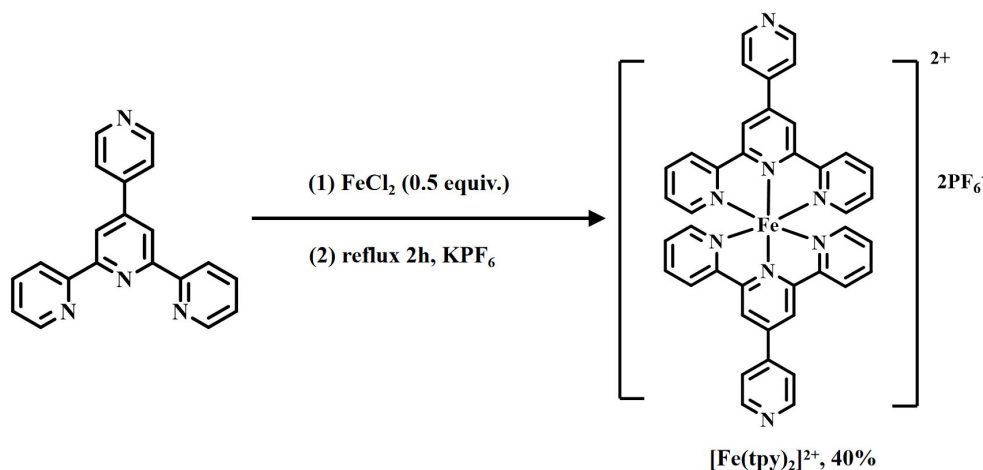
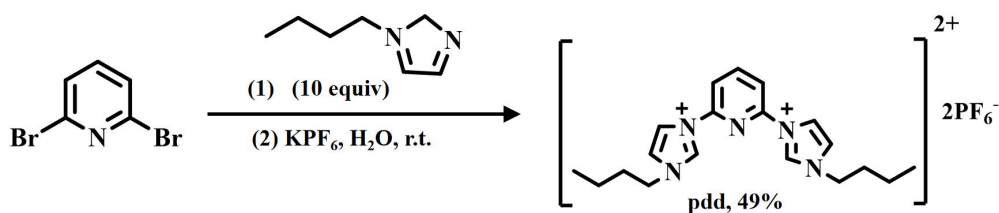
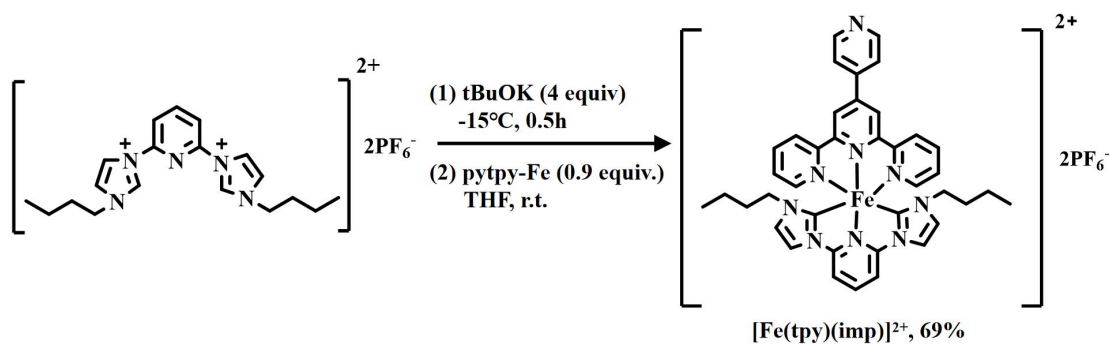
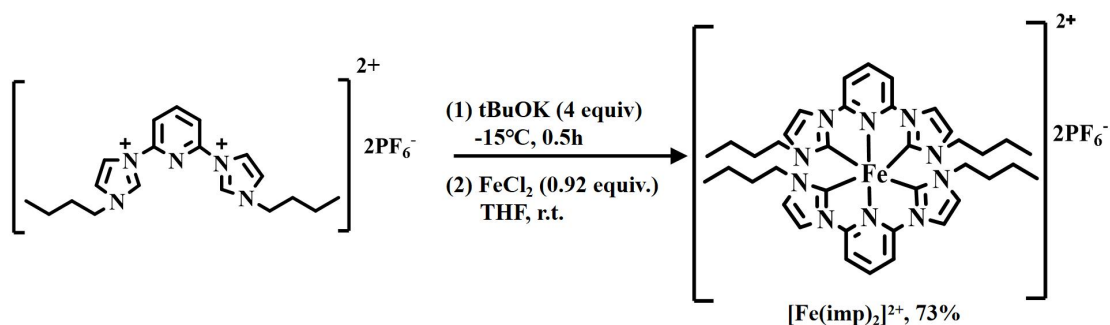
Hep G2 cells are incubated with 10 μM $[\text{Fe}(\text{imp})_2]^{2+}$ (Ex/Em = 800 nm/500-650 nm) for 10 min. Then the cells are further treated with 50 μL Hg^{2+} standard solution (100 $\mu\text{g}/\text{mL}$) and immediately subjected to confocal imaging.

Hep G2 cells are incubated with LPS (2 $\mu\text{g}/\text{mL}$) for 12 hours, followed by the addition of ATP (5 mM) for 1/2 hours.

LPS+ATP1: Hep G2 cells are incubated with ATP (5 mM) for 1 hours.

LPS+ATP2: Hep G2 cells are incubated with ATP (5 mM) for 2 hours.

2.0 Synthesis route of complexes and ligands



Scheme S1 synthesis route of complexes and ligands

pytpy: The pyridine-4-formaldehyde (1.07g, 10 mmol) was dissolved with Ethanol (50 mL) in 250mL round-bottomed flask. Then 2-acetyl pyridine (2.062g, 25mmol) and KOH (2.24g, 40mmol) were added successively. After reacting for one hour, 40ml ammonia water was added in batches and then reflux reaction for four hours. It was further reacted and precipitated solid. The 1.82 g lilac solid (**pytpy**) was obtained with a yield of 59%. ESI-MS m/z : calcd for $C_{20}H_{14}N_4$: 310.12, [M] 310.12. FT-IR (KBr, cm^{-1}): 3455(s), 1600(s), 1353(m), 1108(m), 792(w), 622(m), 505(m). Far-IR (KBr, cm^{-1}): 179(w), 158(w), 133(m), 100(m), 88(w).

pytpy-Fe: The **pytpy** (0.5 g, 0.0016 mol) was dissolved with dichloromethane (10 mL) in 50 mL bottom flask. Adding the solution into anhydrous $FeCl_3$ (0.26 g, 0.0016 mol) dissolved in anhydrous acetonitrile (10 mL), the above mixture stirred at room temperature for 10 min. The yellow solid was collected by filtration and washed with ether and then dried under vacuum to obtain **pytpy-Fe** (0.2g, Yield: 40%). MS calculated for $C_{20}H_{14}Cl_3FeN_4$: 470.96 Found: 434.99[M-Cl-H⁺]. M.p. 308-320°C. IR (KBr, cm^{-1}): 3455.87(m), 1600.65(m), 1353.81(s), 1103.10(s), 792.61(s), 622.91(s), 509.12(s).

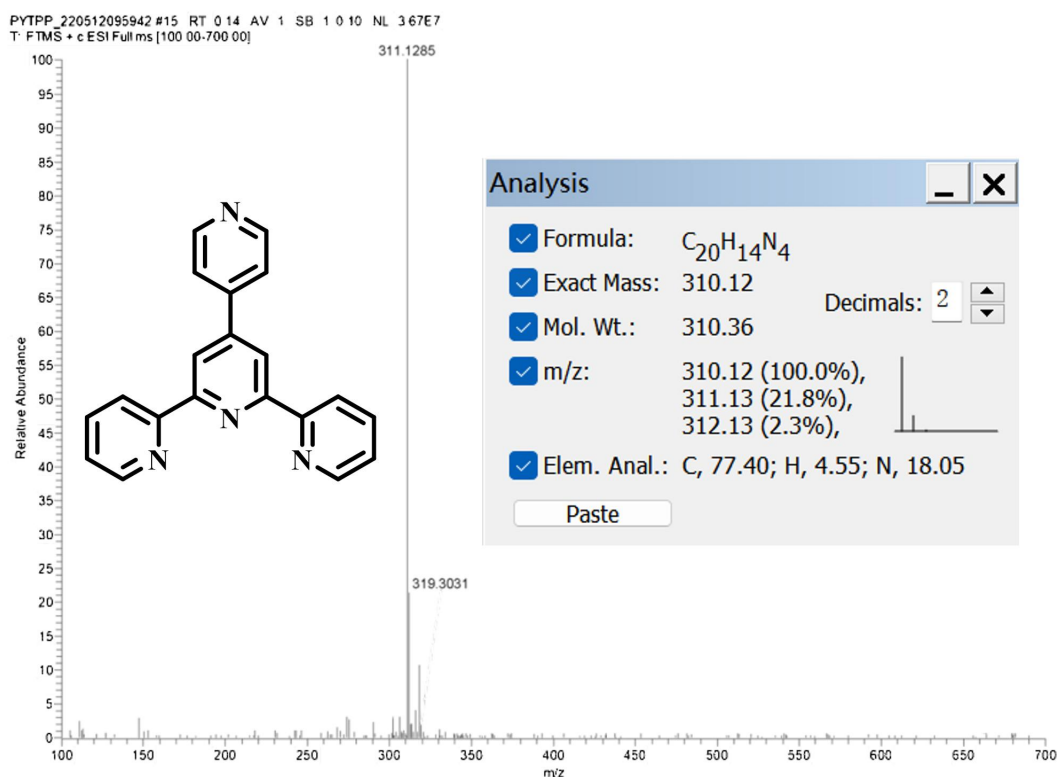
[Fe(tpy)₂]²⁺: The **pytpy** (0.310 g, 0.001 mmol) and acetonitrile (10 mL) were mixed and dissolved. Ethanol (10 mL) and ferrous chloride (0.08 g, 0.0005 mol) were added to the reactants at 80 °C. Reactants were heated to reflux for 2 hours. Then excess KPF_6 were added to the reactants. After the reaction, the purple solid (**[Fe(tpy)₂]²⁺**, 0.14g) was obtained. Yield: 40%. M.p = 362~ 364 °C. ¹H NMR (400 MHz, ACETONITRILE- D_3) δ 9.23 (s, 4H), 9.03 (s, 4H), 8.63 (d, J = 8.0 Hz, 4H), 8.28 (s, 4H), 7.93 (t, J = 7.6 Hz, 4H), 7.19 (d, J = 5.5 Hz, 4H), 7.10 (t, J = 6.6 Hz, 4H). MS calculated for $C_{38}H_{50}FeN_{10}$: 676.18 Found: 338.20 [M/2]. IR (KBr, cm^{-1}) 3410, 1603, 1410, 835.

pdd: The 2,6-dibiomopyridine (9.5 g, 0.04 mol) and 1-butyl-1*H*-imidazole (49.6 g, 0.4 mol) were dissolved in 100 mL Shrek bottle. The mixture was warmed up to 170 °C under nitrogen protection and stirred overnight. After the above mixture was cooled, it was dissolved in methanol (20 ml), then ether (100 ml) was added to disperse it, after which the brown solid was precipitated. The 9.0 g KPF_6 was dissolved in water (100 ml). The above brown solid was dissolved in in water (10 ml), to which the KPF_6 saturated aqueous solution was added dropletly with stirring at room temperature. The yellow-brown solid obtained by filtration was washed with water and dried under vacuum, and then recrystallized with ethanol to obtain 16.6 g white needle-like crystals. Yield: 49%. ESI-MS: cal: M=615.39, found: [M-2PF₆]²⁺/2=162.6. IR (KBr, cm^{-1}): 3453.94(m), 3158.88(s), 2697.96(s), 1612.25(m), 1467.59(s), 1222.67(s), 1120.46(s), 836.97(m), 557.34(m). M.p.: 164-171 °C.

[Fe(tpy)(imp)]²⁺: In a 50ml-Schlenk bottle the ligand **pdd** (0.5 mmol) was suspended in 10 mL of dry THF and cooled down to -15°C. *t*BuOK (2 mL, 1 mol/L in THF) was then added and the mixture was stirred for 30 min at this temperature. The cooling bath was removed and 10 mL THF solution of **pytpy-Fe** (0.45 mmol) was dropped in reaction mixture with a syringe before the mixture was warmed to room temperature. It was then further stirred in the dark overnight and filtered. Then dried under vacuum to obtain

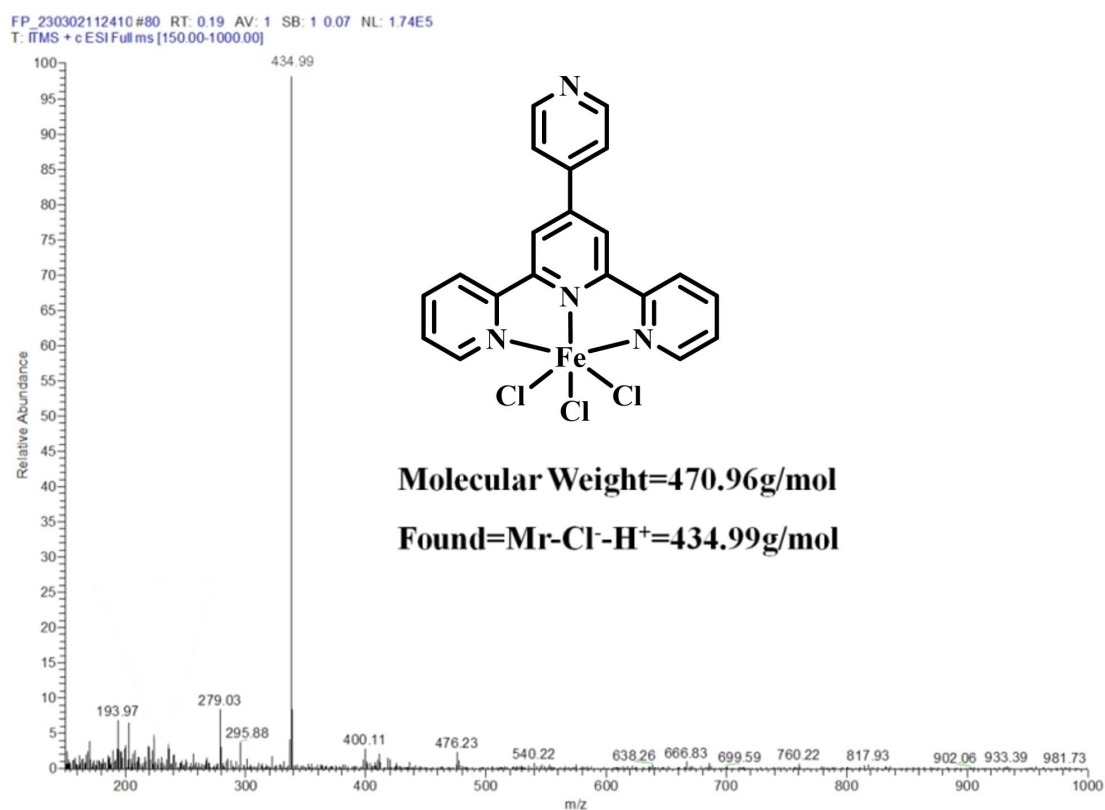
218 $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$: 0.1g, Yield: 69%). ^1H NMR (600 MHz, CD_3CN) δ 9.50 (s, 2H), 9.09 –
 219 8.88 (m, 2H), 8.60 (dd, J = 34.0, 6.8 Hz, 2H), 8.23 (d, J = 9.4 Hz, 2H), 8.12 (d, J = 21.8 Hz,
 220 2H), 8.06 – 7.99 (m, 2H), 7.90 (t, J = 21.2 Hz, 3H), 7.67 (s, 2H), 7.08 (d, J = 20.2 Hz, 2H),
 221 6.96 (s, 2H), 4.30 (s, 4H), 1.42 (d, J = 6.7 Hz, 4H), 0.98 (d, J = 7.0 Hz, 10H). ^{13}C NMR
 222 (151 MHz, CD_3CN) δ 198.17, 160.45, 157.65, 156.67, 153.59, 152.49, 143.76, 138.41,
 223 128.86, 128.05, 126.88, 124.70, 121.30, 120.00, 108.94, 50.39, 34.87, 20.60, 14.08.
 224 ESI-MS calculated for $\text{C}_{39}\text{H}_{39}\text{FeN}_9$: 689.27 Found: 344.6325 $[\text{M}/2]$. M.p.: 319-328°C. IR
 225 (KBr, cm^{-1}): 3448.16(m), 1592.87(s), 1353.81(s), 1106.96(s), 840.83(s), 557.34(s).

226 $[\text{Fe}(\text{imp})_2]^{2+}$: In a 50 ml Schlenk bottle the ligand **pdd** (0.33g, 0.5 mmol) was suspended
 227 in 15 mL of dry THF and cooled down to -15°C . tBuOK (2 mL, 1 mol/L in THF) was then
 228 added and the mixture was stirred for 30 min at this temperature. The cooling bath was
 229 removed and 10 mL THF solution of anhydrous FeCl_2 (0.058g, 0.46 mmol) was dropped
 230 inside with a syringe before the mixture was warmed to room temperature. It was then
 231 further stirred in the dark overnight and filtered. Then dried under vacuum to obtain
 232 $[\text{Fe}(\text{imp})_2]^{2+}$: 0.11g, Yield: 73%). ^1H NMR (600 MHz, CD_3CN) δ 8.22 (s, 2H), 8.09 (s, 4H),
 233 7.79 (s, 4H), 7.11 (s, 4H), 2.62 (s, 8H), 0.81 (s, 8H), 0.66 (s, 20H). ^{13}C NMR (151 MHz,
 234 CD_3CN) δ 198.85, 154.06, 138.85, 125.57, 117.37, 116.90, 105.38, 48.69, 32.79, 19.29,
 235 12.88. MS calculated for $\text{C}_{38}\text{H}_{50}\text{FeN}_{10}$: 702.36 Found: 351.4735 $[\text{M}/2]$. M.p.: 324-330 °C.
 236 IR (KBr, cm^{-1}): 3455.87(m), 2966.03(s), 1592.94(m), 1351.88(s), 1118.53(s), 840.83(m),
 237 557.26(m).



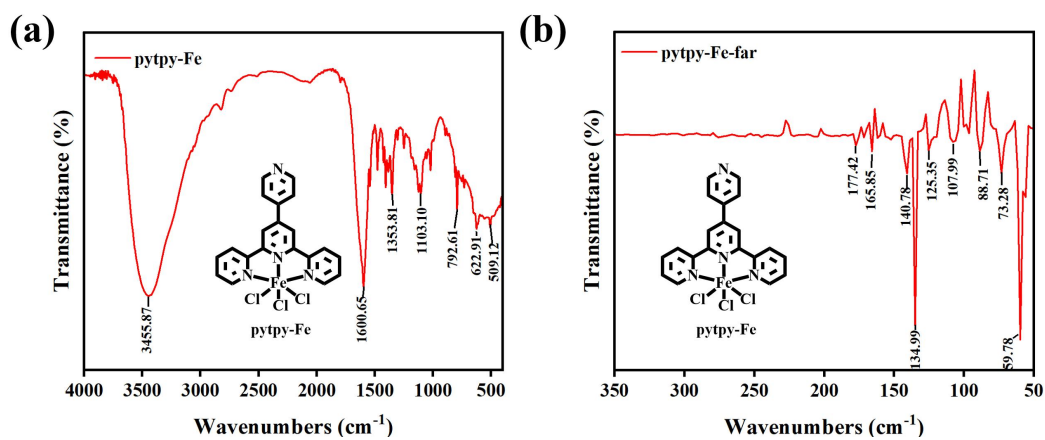
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Figure S1. Mass spectra of pytpy.

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Figure S2. Mass spectra of pytpy-Fe.

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Figure S3. IR spectra of pytpy-Fe.

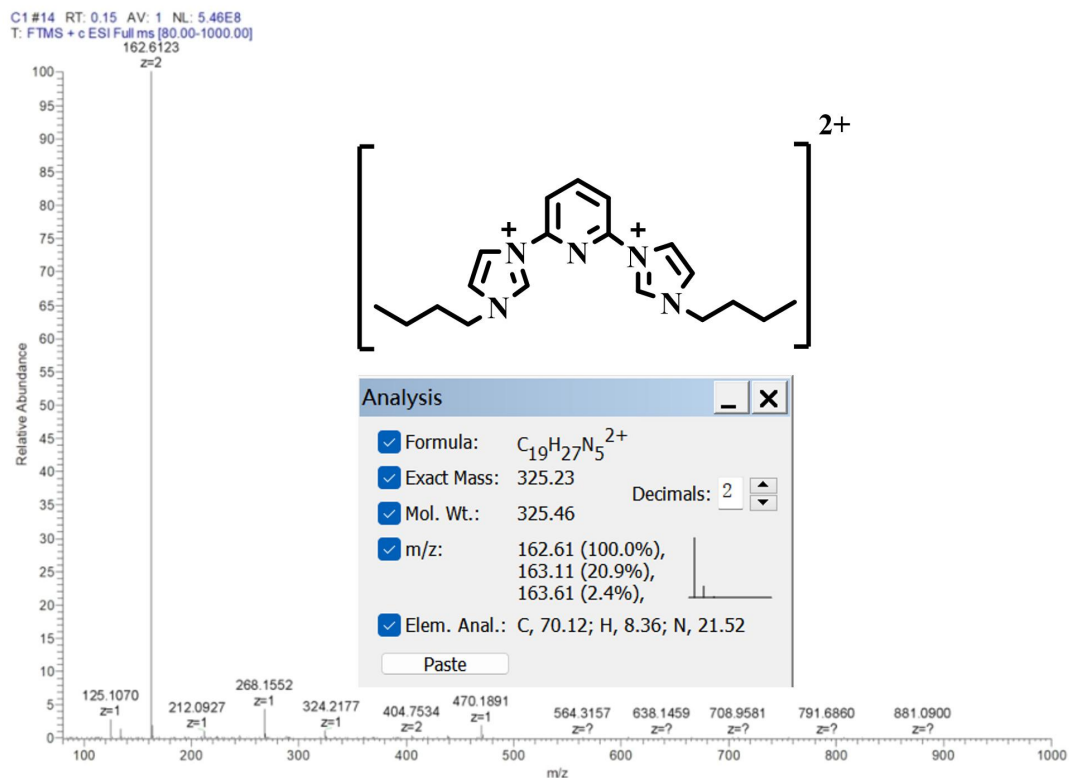


Figure S4. Mass spectra of pdd.

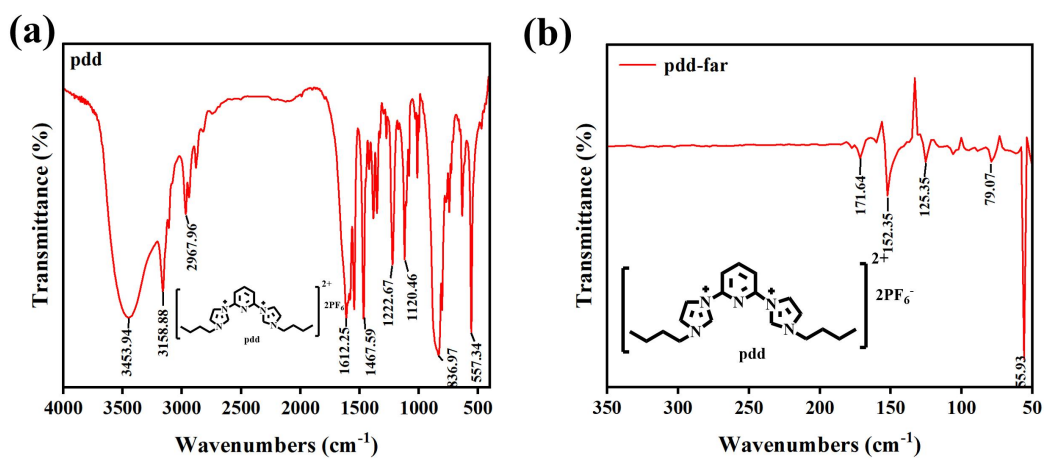


Figure S5. IR spectra of pdd.

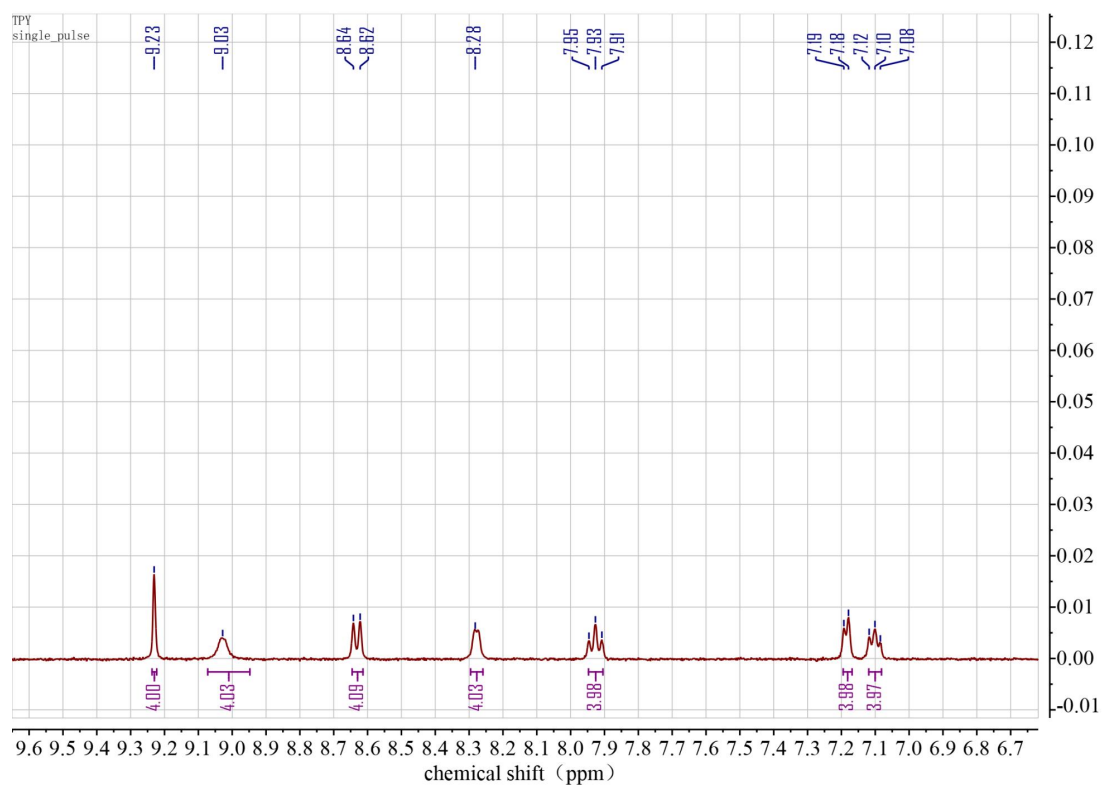


Figure S6. ^1H NMR spectra of $[\text{Fe}(\text{tpy})_2]^{2+}$ (600 MHz, CD_3CN).

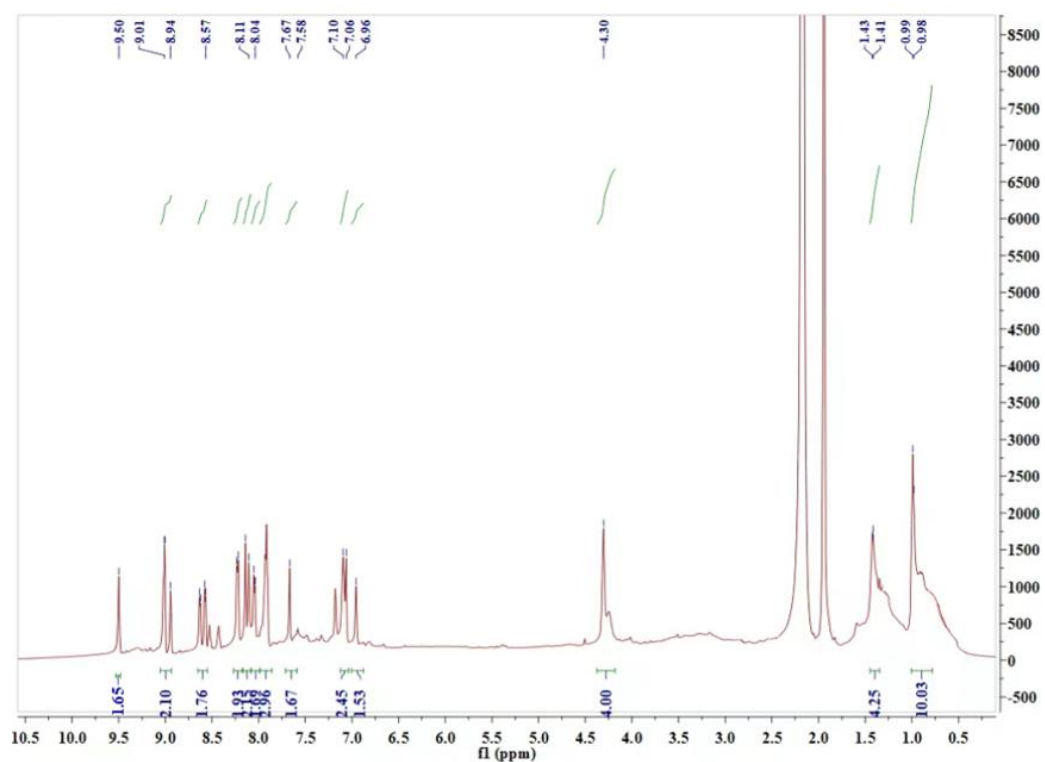


Figure S7. ^1H NMR spectra of $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$ (600 MHz, CD_3CN).

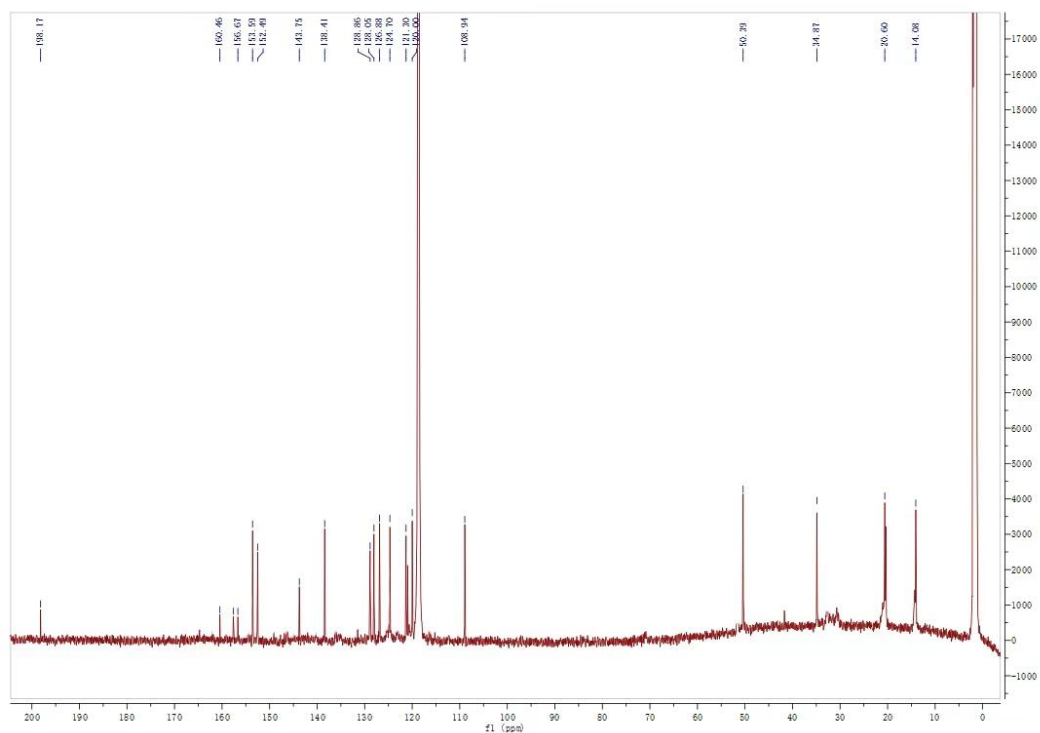


Figure S8. ^{13}C NMR spectra of $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$ (151 MHz, CD_3CN).

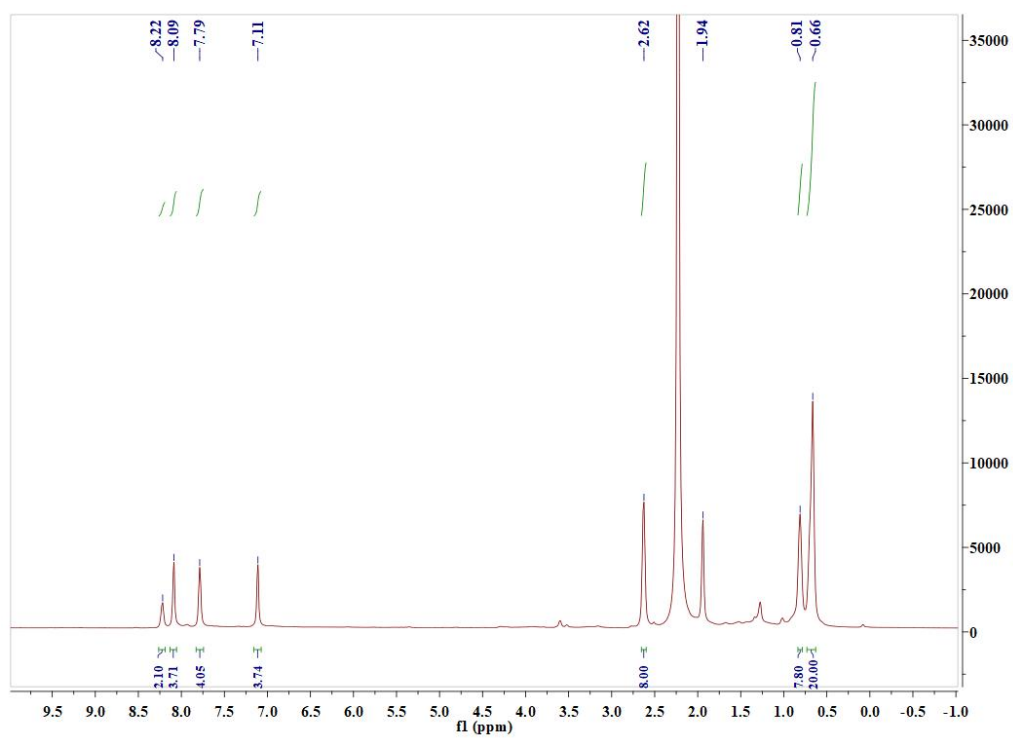


Figure S9. ^1H NMR spectra of $[\text{Fe}(\text{imp})_2]^{2+}$ (600 MHz, CD_3CN).

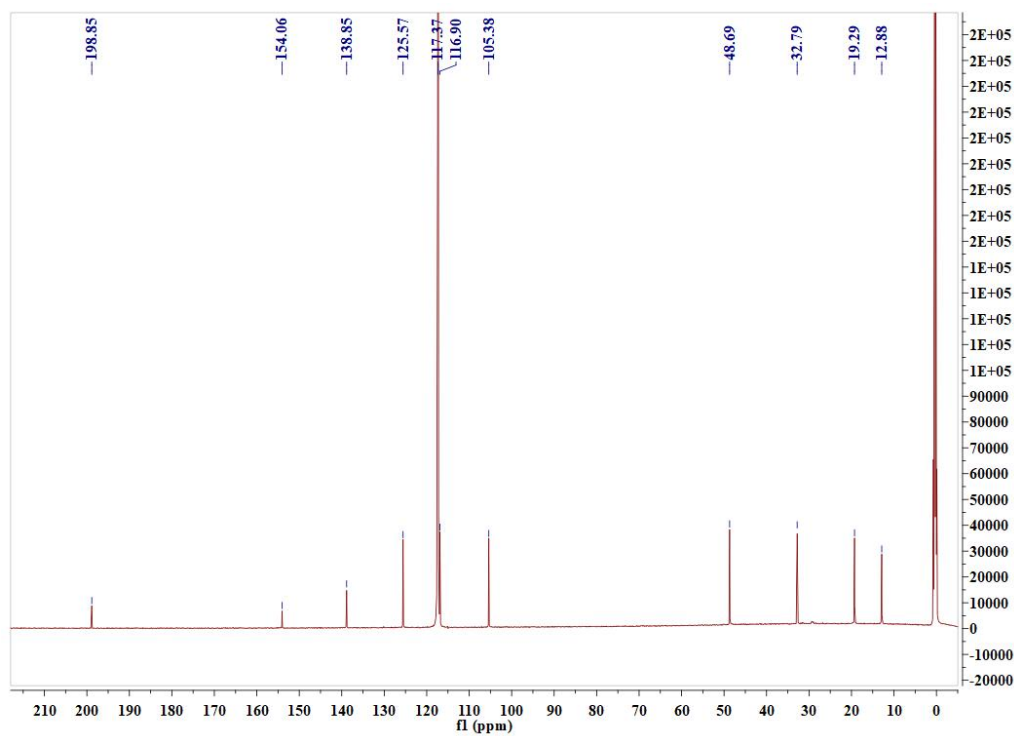


Figure S10. ^{13}C NMR spectra of $[\text{Fe}(\text{imp})_2]^{2+}$ (151 MHz, CD_3CN).

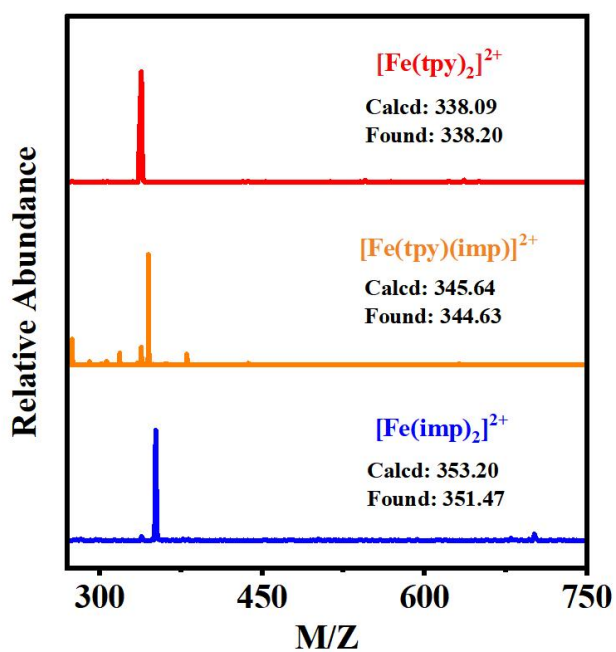


Figure S11. Mass spectra of $\text{Fe}(\text{tpy})_2]^{2+}$, $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$ and $[\text{Fe}(\text{imp})_2]^{2+}$.

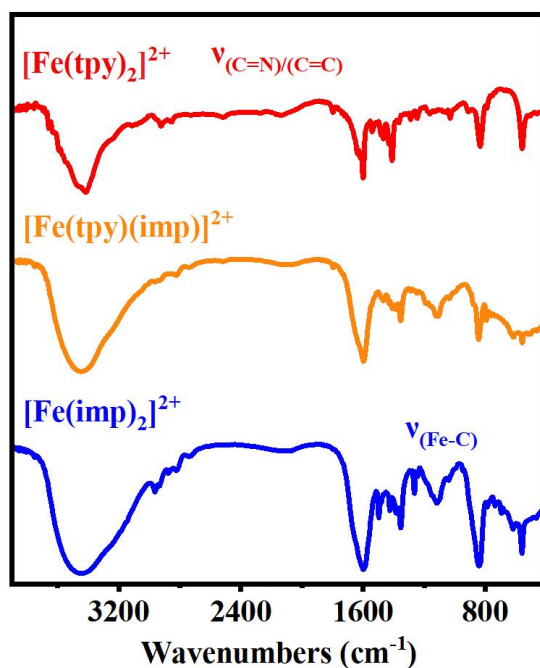


Figure S12. IR spectra of $[\text{Fe}(\text{tpy})_2]^{2+}$, $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$ and $[\text{Fe}(\text{imp})_2]^{2+}$.

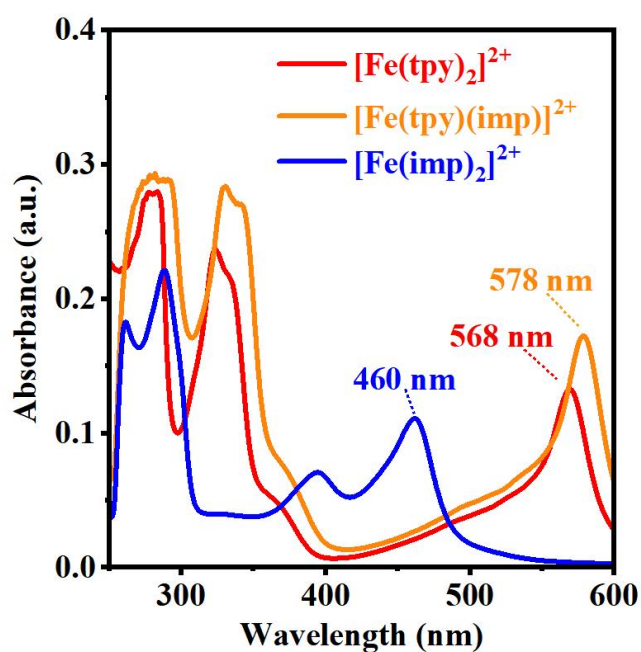


Figure S13. UV-Vis spectra of $[\text{Fe}(\text{tpy})_2]^{2+}$, $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$ and $[\text{Fe}(\text{imp})_2]^{2+}$.

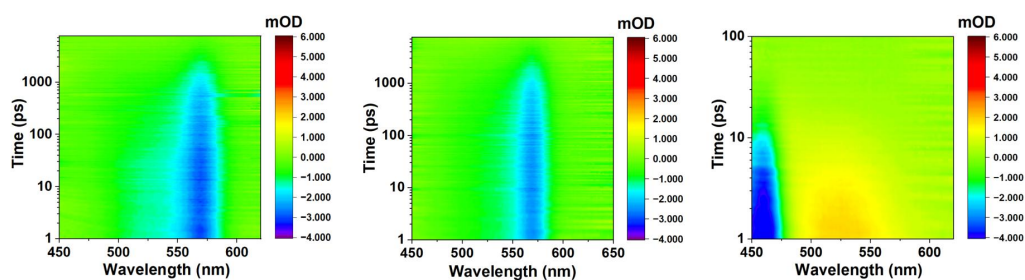


Figure S14. Femtosecond transient absorption spectra of $[\text{Fe}(\text{tpy})_2]^{2+}$, $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$ and $[\text{Fe}(\text{imp})_2]^{2+}$

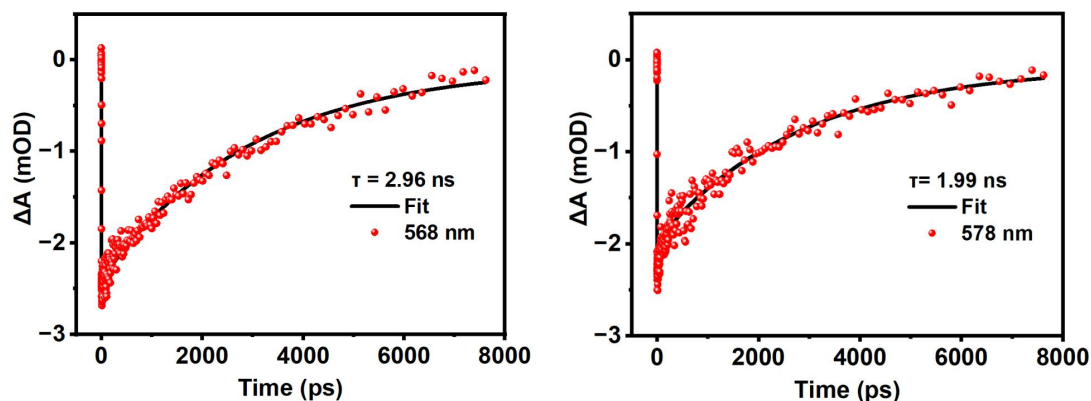


Figure S15. Kinetic trace curve of $[\text{Fe}(\text{tpy})_2]^{2+}$, $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$

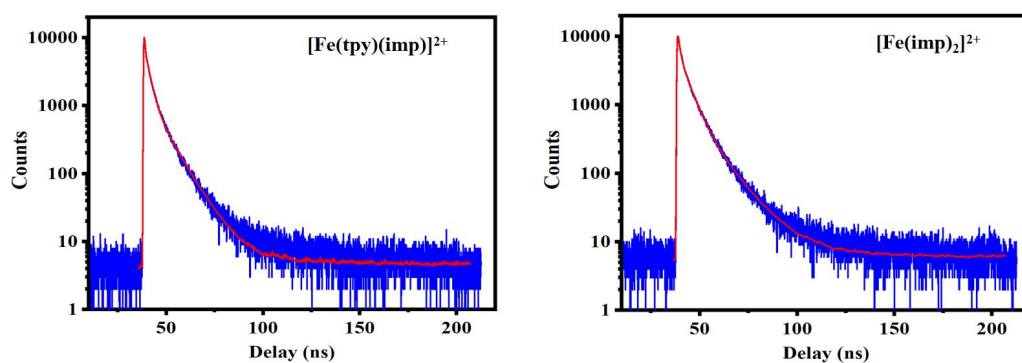


Figure S16. Fluorescence lifetime of $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$ and $[\text{Fe}(\text{imp})_2]^{2+}$

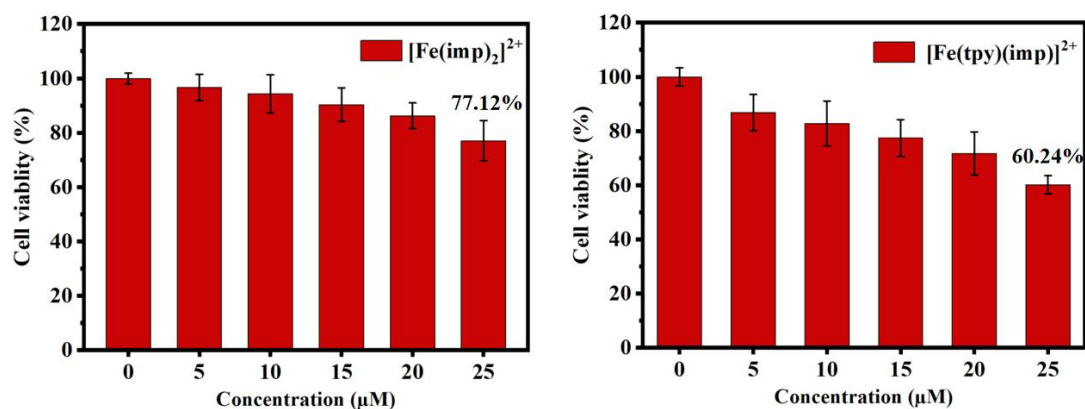


Figure S17. Cytotoxicity of $[\text{Fe}(\text{imp})_2]^{2+}$ and $[\text{Fe}(\text{tpy})(\text{imp})]^{2+}$

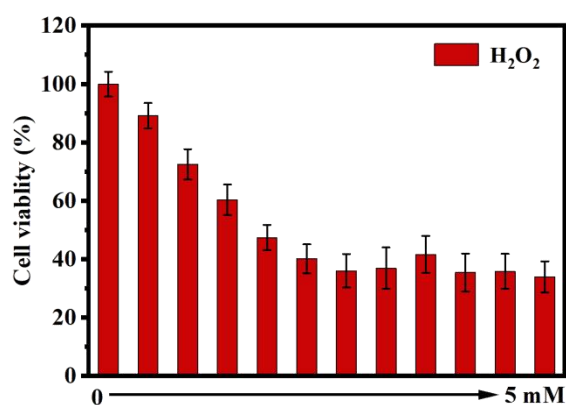


Figure S18. H₂O₂ induced apoptosis model

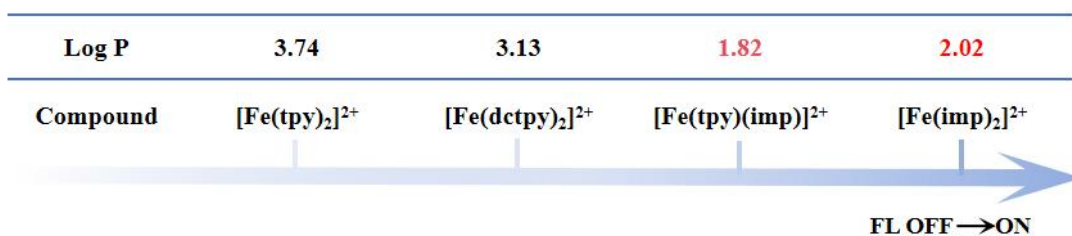


Figure S19. Partition Coefficient of [Fe(tpy)₂]²⁺, [Fe(dctpy)₂]²⁺, [Fe(tpy)(imp)]²⁺ and [Fe(imp)₂]²⁺

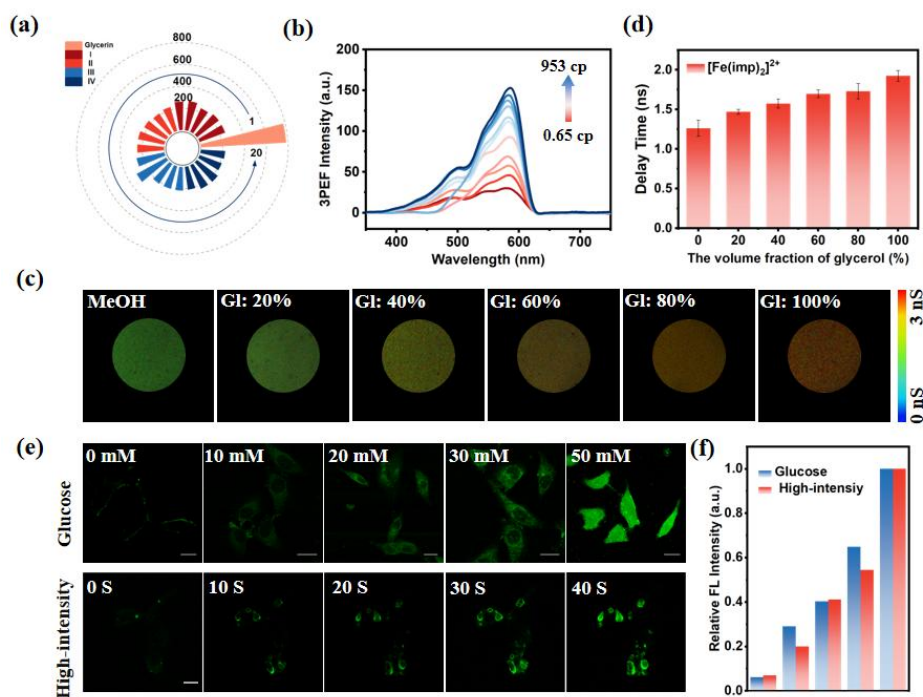


Figure S20. Viscosity response test of [Fe(imp)₂]²⁺ (a) Selectivity data of [Fe(imp)₂]²⁺ (1×10⁻⁵ mol/L). (b) Three-photon fluorescence spectra of [Fe(imp)₂]²⁺ in different viscous environments. (c) Confocal fluorescence lifetime imaging of [Fe(imp)₂]²⁺ in different

viscous solution environment. (d) Quantitative Chart of Figure S20c. (e) Confocal two-photon fluorescence imaging $[\text{Fe}(\text{imp})_2]^{2+}$. Hep G2 cells were treated with different glucose concentrations or exposed to intense laser irradiation (EX: 800 nm, EM: 500-650 nm) (f) Quantified fluorescence intensity of Figure S20e.

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