Supporting Information

A Ratiometric pH Probe for Acidification Tracking in Dysfunctional Mitochondria and Tumour Tissue *in vivo*

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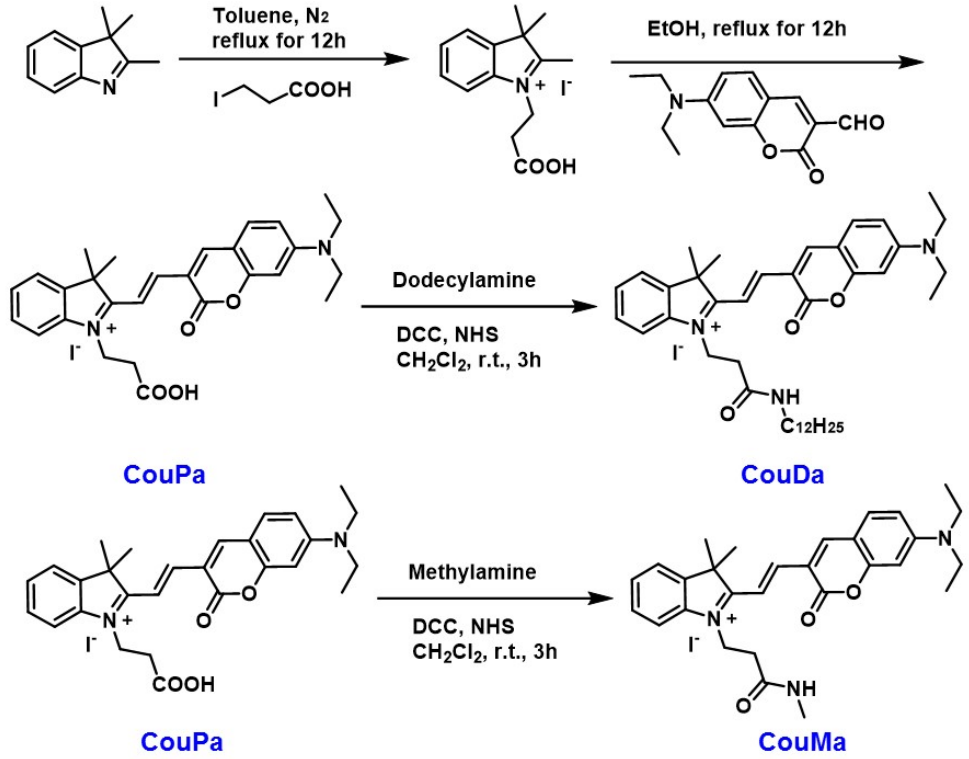
1. Materials and general methods

All the reagents and solvents were purchased from commercial source including Alfa Aesar, JK chemical, and Energy Chemical. They were of analytic grade and used without purification. MitoTracker Deeep Red 633 was purchased from Invitrogen. MEM media was purchased from Jiangsu Keygen BioTech Co. Ltd. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DRX-400 with TMS as internal reference. High resolution mass spectrometric data were determined with an Agilent 6540Q-TOF LC/MS mass spectrometer, while ESI mass spectrometric data were collected with a Thermo Fisher ESI Mass Spectrometer. Fluorescence tests were performed on FluoroMax-4 Spectrofluorometer with 5 nm slit for both excitation and emission. Absorption spectra were measured on a Perkin Elmer Lambda 35 spectrophotometer. All pH measurements were accomplished by a Model PHS-3C meter. Confocal imaging of MCF-7 cells was realized using confocal microscope Zeiss LSM710. Optical imaging of mice was carried out on PerkinElmer IVIS Lumina K Series III in vivo imaging system.

2. Synthesis and characterization of CouDa and CouMa



Scheme S1. Synthesis of CouDa and CouMa.

Synthesis of probe CouDa

CouPa was obtained according to the procedure reported previously[1]. CouPa (1.92 mM, 1.126 g), DCC (1.92 mM, 0.396g) and NHS (1.92 mM, 0.221 g) were dissolved in CH_2Cl_2 (40 mL) and activated under 0°C for 0.5 h. After that dodecylamine (2.3 mM, 0.427 g) was added and the solution was stirred at room temperature for nearly 2.5 h, which was monitored by TCL. When the reaction was finished, cool the solution under 4°C overnight and remove the precipitate by filtration. Solvent of filtrate was removed under reduced pressure, and the crude product was purified by silica gel chromatography with eluent CH_2Cl_2/CH_3OH (1/40, v/v) to afford CouDa as a blue solid (0.72 g). Yield, 50%.

^{1}H NMR (400 MHz, CD_3OD, δ, ppm): 8.60 (s, 1H), 8.34 (d, J = 15.7 Hz, 1H), 7.97 (d, J = 15.7 Hz, 1H), 7.73 (m, 2H), 7.66 – 7.52 (m, 3H), 6.93 (dd, J = 9.1, 2.3 Hz, 1H), 6.65 (d, J = 2.1 Hz, 1H), 4.79 (t, J = 6.3 Hz, 2H), 3.62 (q, J = 7.1 Hz, 4H), 3.01 (t, J = 7.3 Hz, 2H), 2.85 (t, J = 6.3 Hz, 2H),1.83 (s, 6H), 1.36 – 1.08 (m, 26H), 0.89 (t, J = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD, δ, ppm): 181.86, 169.60, 160.07, 158.20, 154.77, 150.40, 149.49, 143.09, 140.69, 132.40, 128.87, 128.55, 122.44, 114.01, 112.32, 111.25, 110.10, 109.57, 96.43, 51.66, 45.03, 42.79, 39.23, 33.60, 31.57, 29.23, 29.18, 29.11, 28.96, 28.91, 28.74, 26.45, 25.78, 22.23, 12.94, 11.35. HR-MS (positive mode, *m/z*): calcd. 626.4316, found 626.4319 for $[M]^+$.

Synthesis of CouMa

CouMa was obtained with a procedure similar to CouDa. The crude product was purified by silica gel chromatography with eluent CH_2Cl_2/CH_3OH (1/20, v/v) to afford CouMa as a dark blue solid. Yield, 65%.

^{1}H NMR (400 MHz, CD_3OD, δ, ppm): 8.65 (s, 1H), 8.33 (d, 1H, J = 15.7Hz), 7.95 (d, 1H, J = 15.6 Hz), 7.76-7.70 (m, 2H), 7.63-7.55 (m, 3H), 6.90 (d, 1H, J = 9.1 Hz), 6.61 (s, 1H), 4.79 (t, 2H, J = 6.6 Hz), 3.61 (q, 4H, J = 7.1 Hz), 2.86 (t, 2H, J = 6.7 Hz), 2.59 (s, 3H), 1.82 (s, 6H), 1.26(t, 6H, J = 7.1 Hz).^{13}C NMR (101 MHz, CD_3OD, δ, ppm): 183.42, 171.76, 169.12, 161.66, 159.71, 156.27, 151.99, 144.63, 142.21, 133.95, 130.39, 130.06, 123.99, 115.48, 113.82, 112.76, 111.62, 111.04, 97.94, 53.22,46.58, 44.33, 34.91, 27.33, 26.47, 12.90. HR-MS (positive mode, *m/z*): calcd. 472.2599, found 472.2595 for $[M]^+$.

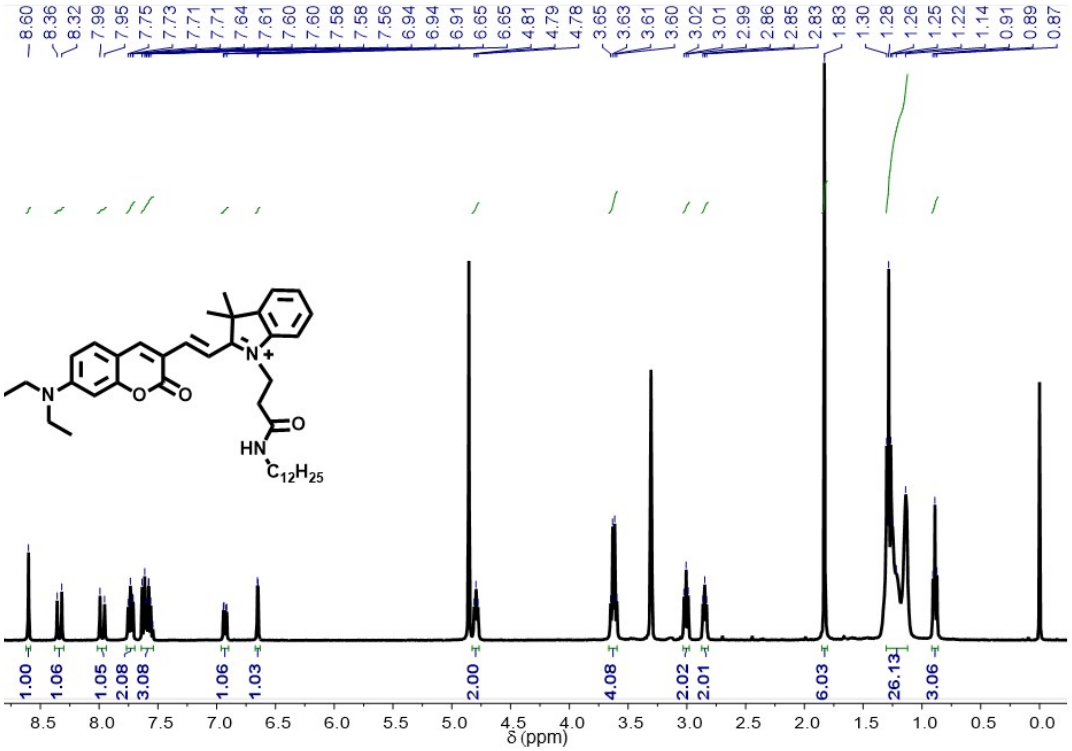


Figure S1. ^{1}H NMR spectrum of CouDa in CD_3OD.

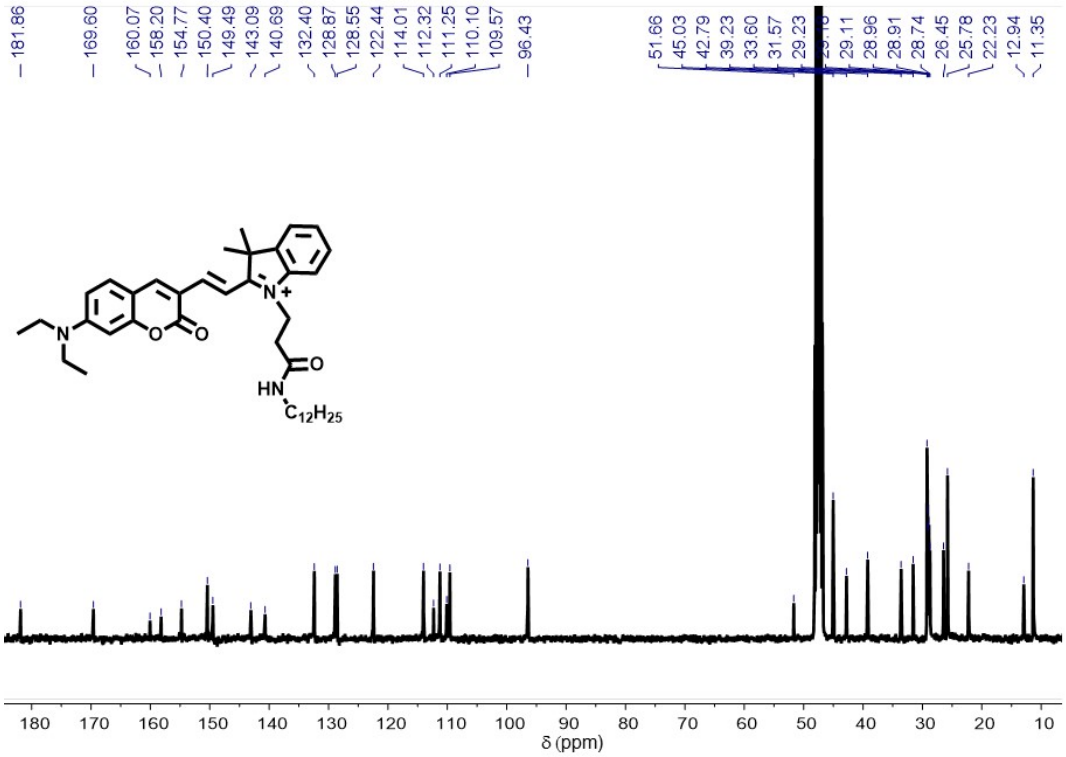


Figure S2. ^{13}C NMR spectrum of CouDa in CD_3OD.

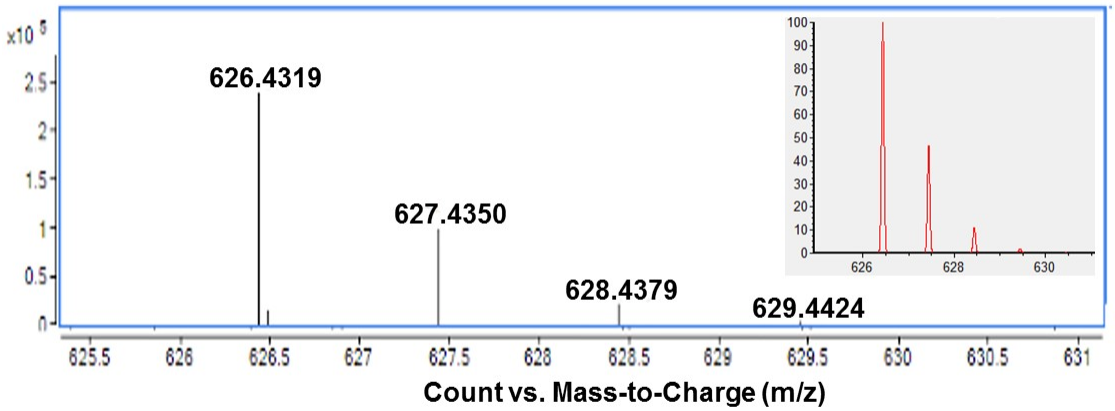


Figure S3. HR-MS spectrum of CouDa. Inset: Simulated isotopic distribution pattern of CouDa.

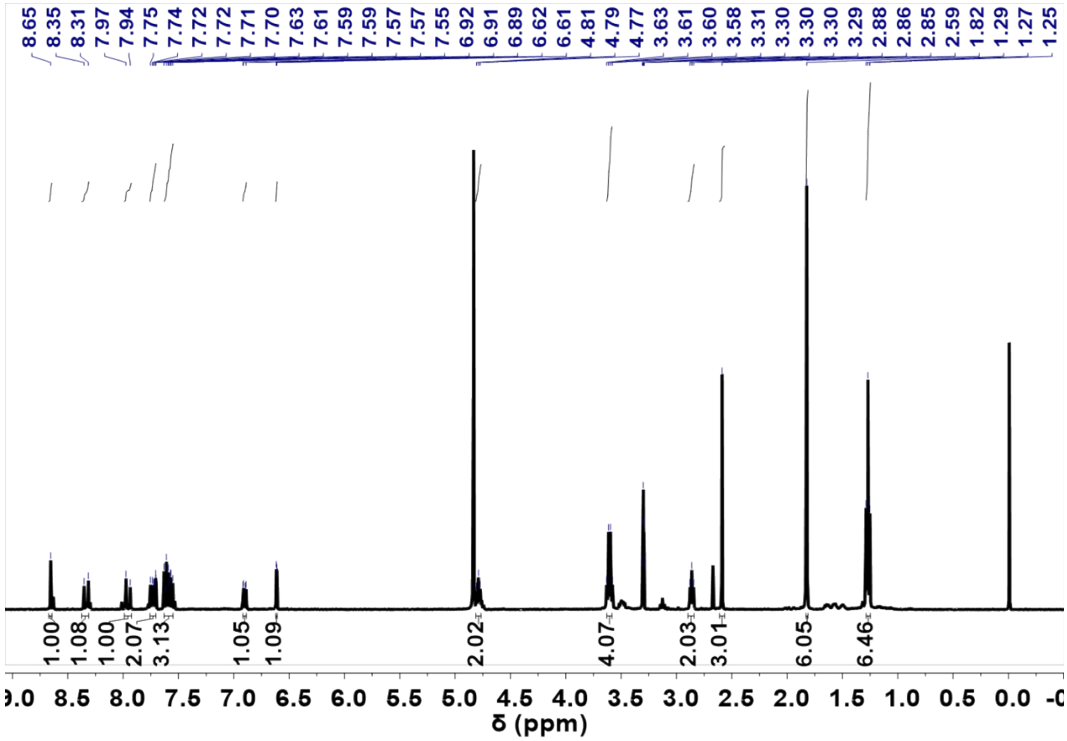


Figure S4. ^{1}H NMR spectrum of CouMa in CD_3OD.

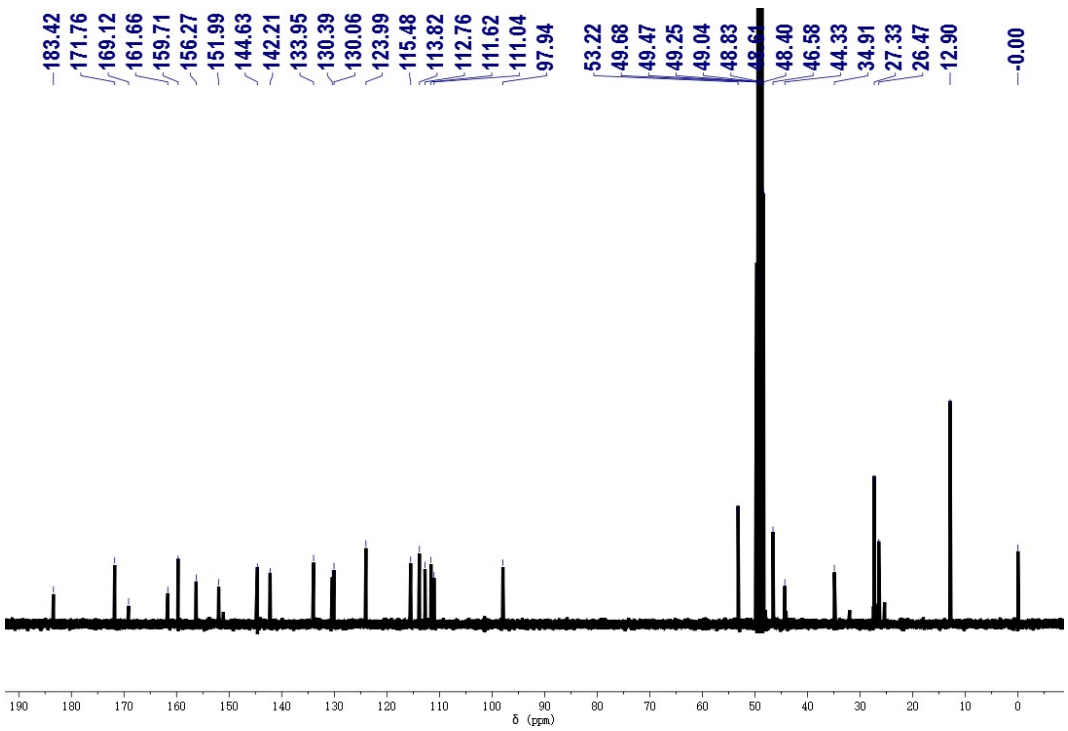


Figure S5. ^{13}C NMR spectrum of CouMa in CD_3OD.

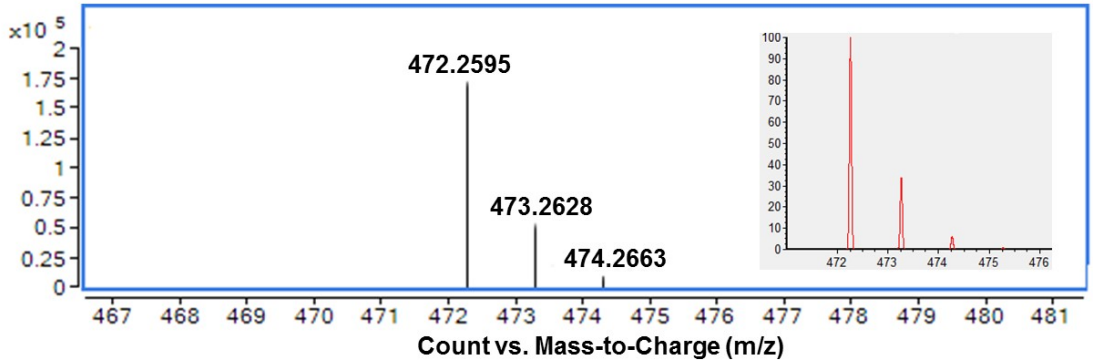


Figure S6. HR-MS spectrum of CouMa. Inset: Simulated isotopic distribution pattern of CouMa.

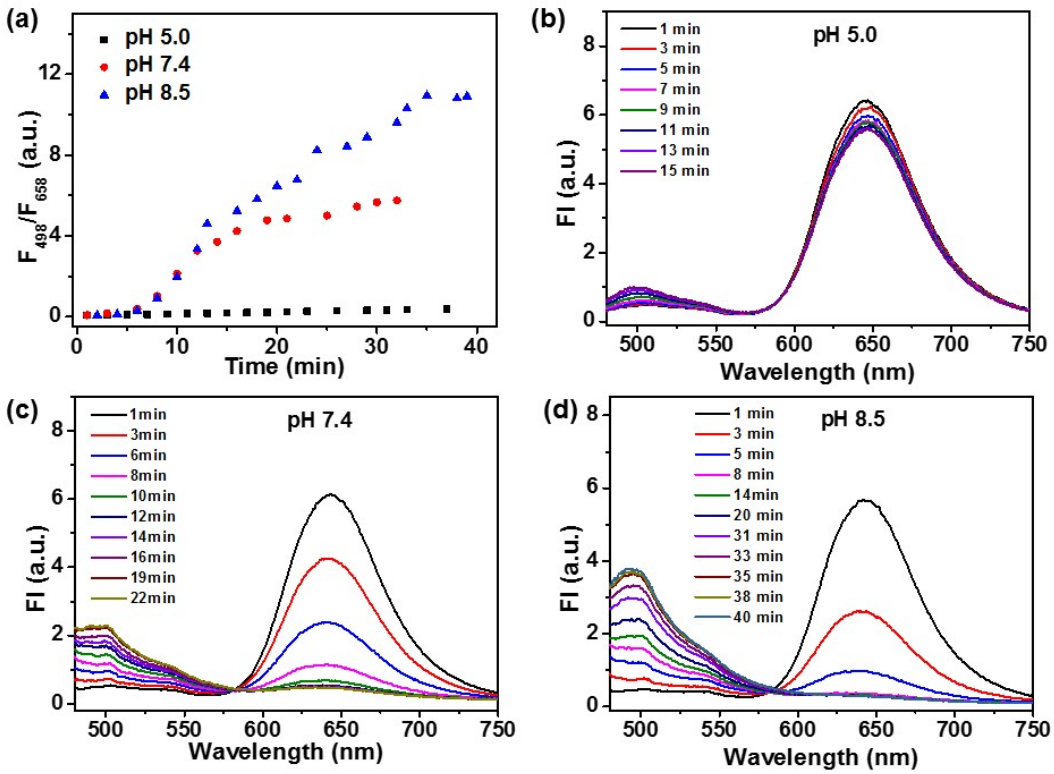


Figure S7. (a) The temporal profile of CouDa fluorescence ratio (F_{498}/F_{658}) in Na_2HPO_4-citric acid buffer solution with pH 5.0, 7.4 and 8.5, respectively. (b, c, d) Time-dependent fluorescent emission spectra of CouDa (10 μM, 10% DMSO, v/v) in Na_2HPO_4-citric acid buffer solution with pH 5.0 (b), 7.4 (c) and 8.5 (d), respectively.

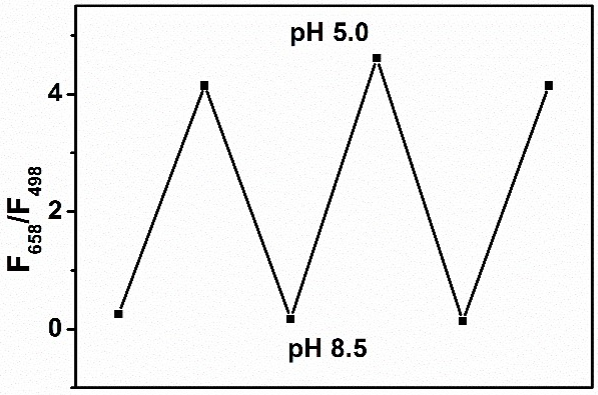


Figure S8. Emission ratio of CouDa recorded when pH changed between 8.5 and 5.0 circularly.

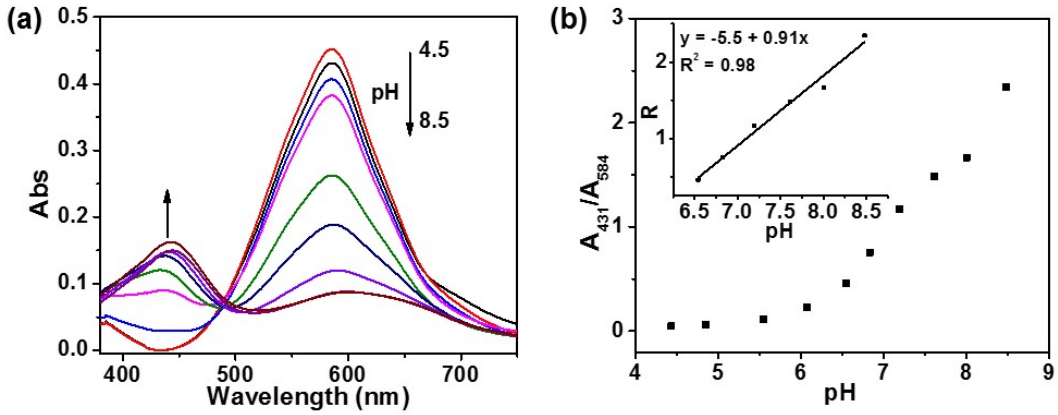


Figure S9. (a) Absorption spectra of CouDa (10 μM, 10% DMSO, v/v) in Na_2HPO_4-citric acid buffer solution with pH ranging from 4.5 to 8.5. (b) Absorption intensity ratio (A_{431}/A_{584}) of CouDa versus pH. Inset: Linear relationship of pH versus absorption intensity ratio. λ_{ex}, 460 nm. Spectra were collected after 40 min when saturated by ambience.

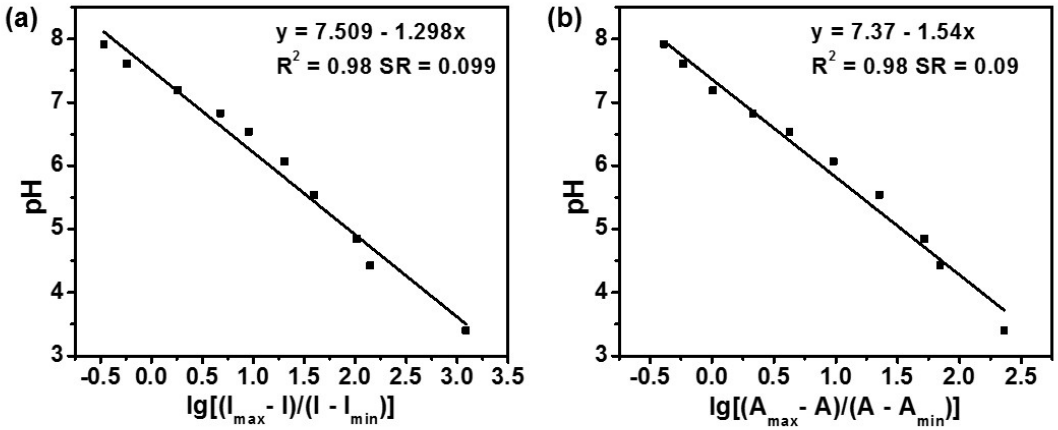


Figure S10. Linear fitting of pH versus $lg[(I_{max}–I)/(I–I_{min})]$ (a) or pH versus $lg[(A_{max}–A)/(A–A_{min})]$ (b), where I is the ratio of F_{498}/F_{658}, and A is the ratio of A_{431}/A_{584}. Solution of CouDa (10 μM, 10% DMSO, v/v) in Na_2HPO_4-citric acid buffer was addopted. λ_{ex}, 460 nm.

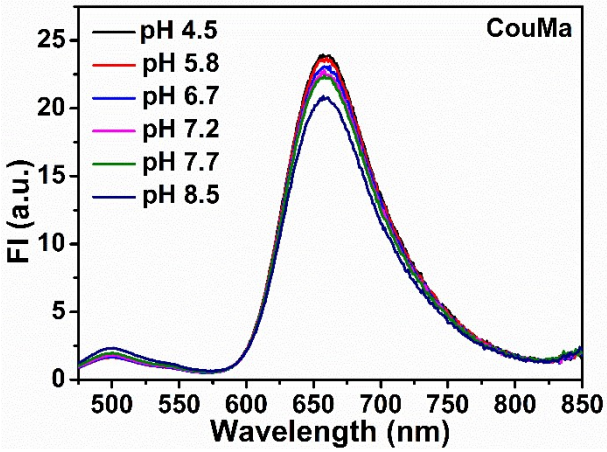


Figure S11. Emission spectra of CouMa (10 μM, 10% DMSO, v/v) in Na_2HPO_4-citric acid buffer solution with pH ranging from 4.5 to 8.5. λ_{ex}, 460 nm.

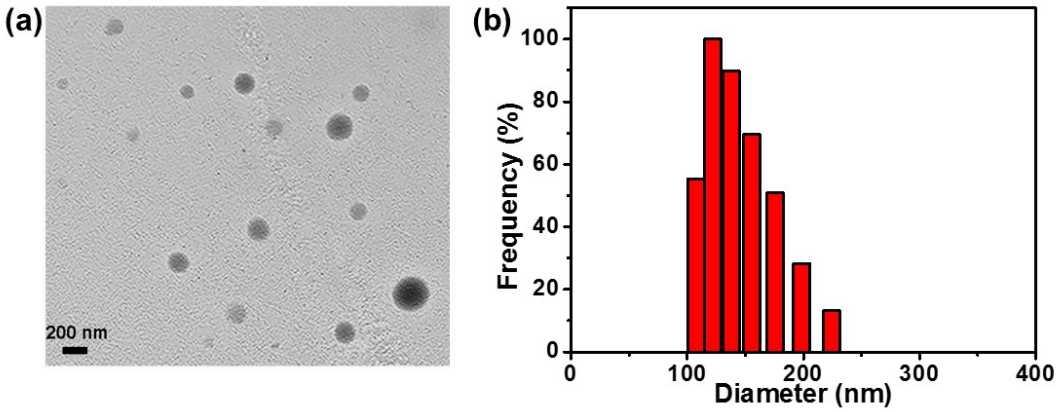


Figure S12. (a) TEM image of CouDa micelles in Na_2HPO_4-citric acid buffer solution (10 μM, 10% DMSO, v/v). (b) DLS determination for the diameter of CouDa micelles in the same aqueous solution as TEM test.

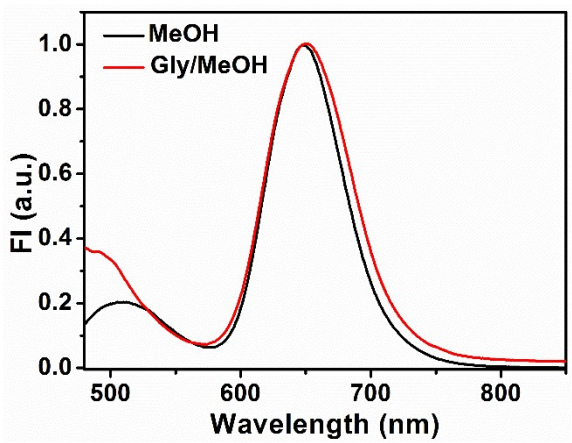
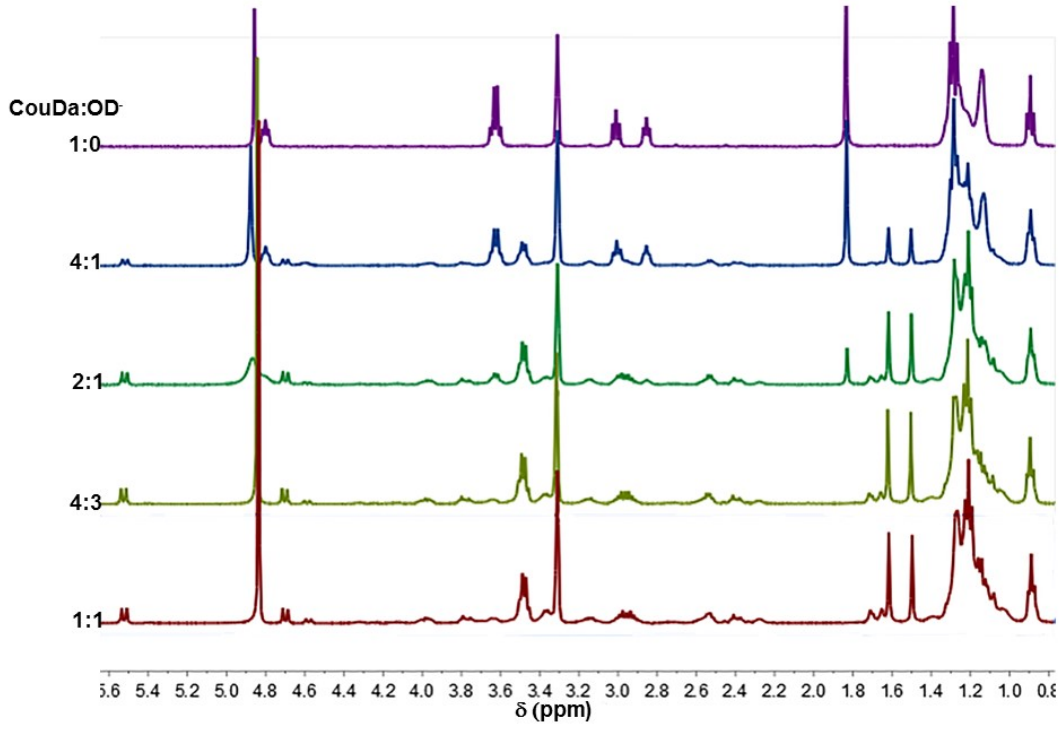


Figure S13. Fluorescent emission spectra of CouDa in methanol and glycerol/methanol mixture solution (9/1, v/v). λ_{ex}, 460 nm.



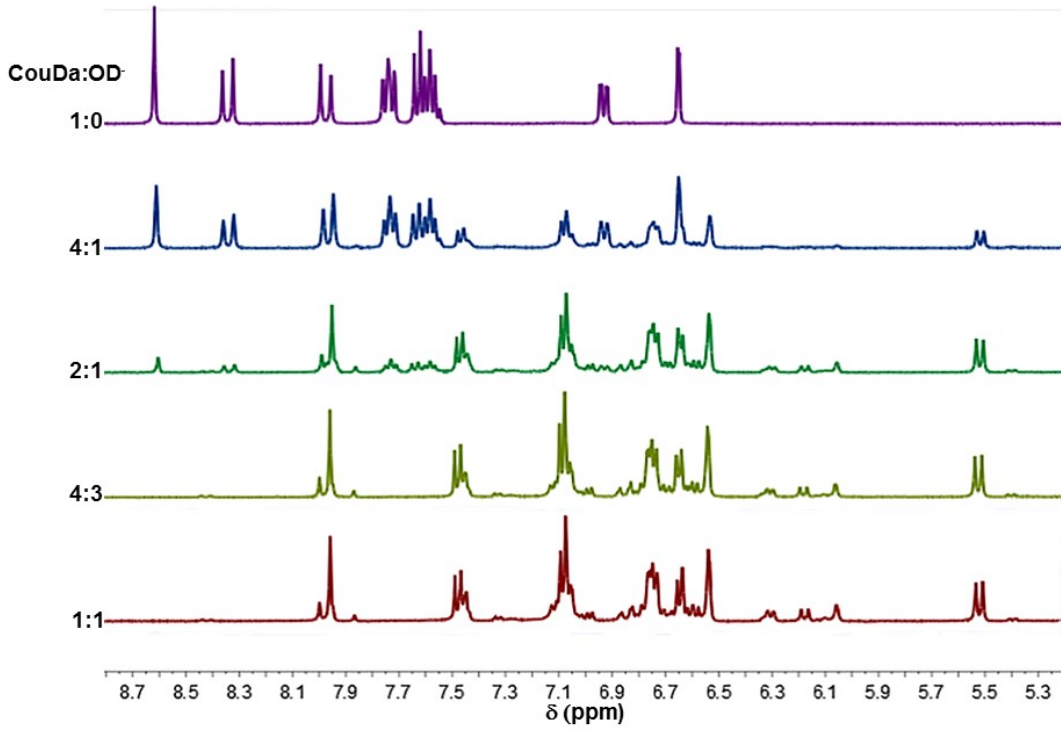


Figure S14. ^{1}H NMR spectra of CouDa (17 mM in CD_3OD) when different amount of NaOD (0.2 M in D_2O) was added.

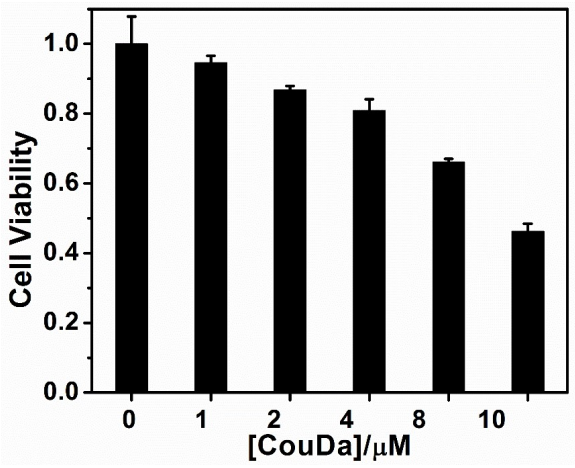


Figure S15. MCF-7 cell viability after incubated with different concentrations of CouDa for 12 h, measured by MTT assay.

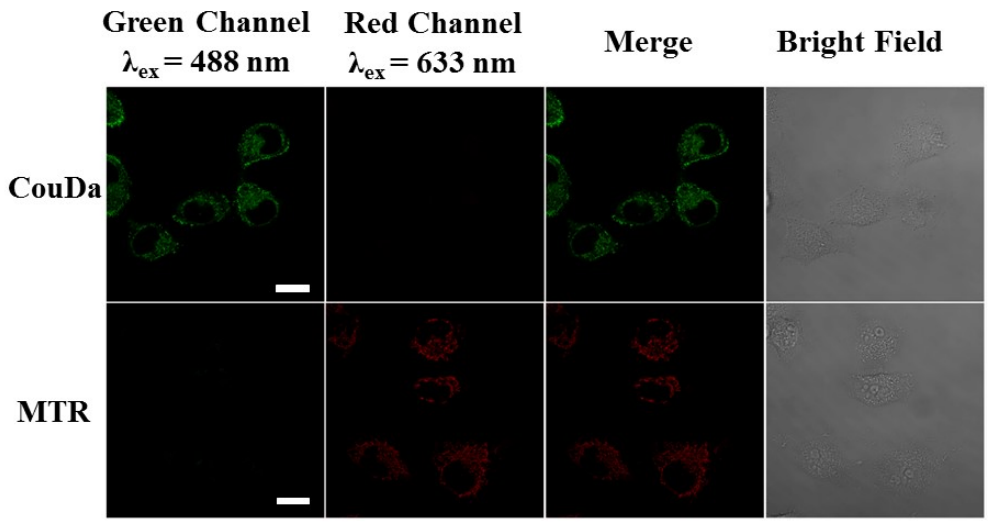


Figure S16. Confocal fluorescence images of MCF-7 cells treated with CouDa (2 μM, 1% DMSO, v/v) and MitoTracker Deep Red 633 (MTR, 0.2 μM), respectively. Green channel, λ_{ex} = 488 nm, λ_{em} = 492-700 nm; Red channel, λ_{ex} = 633 nm, λ_{em} = 640-750 nm; Merge channel image was obtained by overlapping the green channel with corresponding red channel. Scale bar, 20 μm.

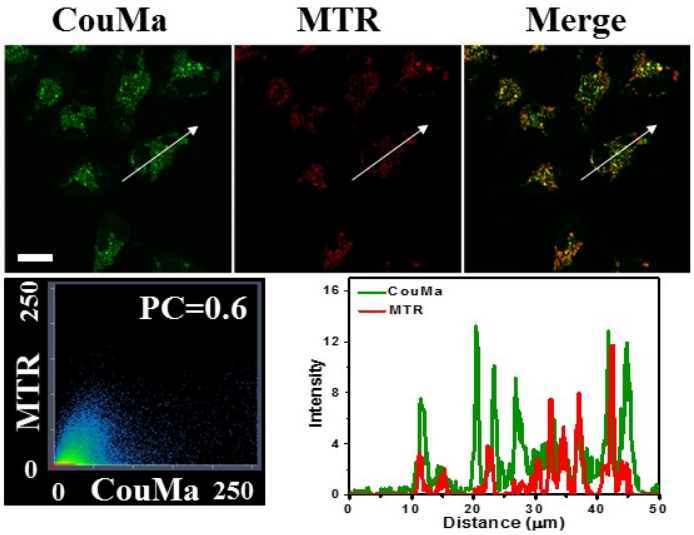


Figure S17. Confocal fluorescence images of MCF-7 cells costained with CouMa (2 μM, 1% DMSO, v/v) and MitoTracker Deep Red 633 (MTR, 0.2 μM). Fluorescence profiles along the white arrow from the green and red channels are shown. Green channel, λ_{ex} = 488 nm, λ_{em} = 492-700 nm; Red channel, λ_{ex} = 633 nm, λ_{em} = 640-750 nm. Scale bar, 20 μm.

References

1 Y. Zhang, Y. Chen, H. Fang, X. Shi, H. Yuan, Y. Bai, *Analyst*, 2020, **145**, 5123-5127.