Supporting Information for

Title: A novel near-infrared ratiometric fluorescent probe capable of copper(II) ion determination in living cells

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Contents

1. Materials and Instruments
2. Synthesis and Characterization of CR-Ac
3. Cell Culture and Confocal Imaging
4. Supplementary Figures (S1-S16)
5. Calculation of Quantum Yield
6. Calculation of Binding Constant of CR-Ac with Cu\textsuperscript{2+}
7. References
1. Materials and Instruments

4-diethylamino-salicylaldehyde, ethyl acetoacetate, hydrazine monohydrate, and benzotriazol-1-yloxytris (dimethylamino)-phosphonium hexafluorophosphate (BOP) were purchased from TCI America. Piperidine was purchased from Sigma-Aldrich. MitoTracker Green FM, LysoTracker Blue DND-99, and the nuclear stain Hoechst 33258 were purchased from Life Technologies and used in accordance with the manufacturer’s protocols. The other chemicals and the solvents used in the experiments were purchased commercially. Solutions of metal ions of Ni\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Pb\(^{2+}\), Cr\(^{3+}\), Hg\(^{2+}\), Mn\(^{2+}\), Ag\(^{+}\), Co\(^{2+}\), K\(^{+}\), Na\(^{+}\), Ca\(^{2+}\) and Mg\(^{2+}\) were prepared by dissolving their chloride, sulfate or nitrate salts into double-distilled water while those for Fe\(^{2+}\), Fe\(^{3+}\) were freshly prepared in 0.01 M HCl. Solution of Cu\(^{+}\) was freshly prepared by dissolving tetrakis(acetonitrile)copper(I) (Sigma-Aldrich) into double-distilled water.

\(^{1}\)H and \(^{13}\)C NMR spectra were recorded on a Bruker AVANCE III HD 400 at ambient temperature (298K). Chemical shifts are reported in delta (\(\delta\)) unit per million (ppm) downfield tetramethylsilane. Splitting patterns are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. ESI-MS analyses were performed on a Perkin Elmer API 150 EX mass spectrometer or a Waters ACQUITY-Xevo Qtof UPLC mass spectrometer. UV/Vis spectra were recorded on a Perkin Elmer Lambda 25 spectrometer at 293 K. Fluorescence spectra were recorded on a Perkin Elmer LS55 luminescence spectrometer at 293 K. The excitation wavelengths and filters used were indicated in the figures. The relative quantum yield was measured by using a Fluoromax-2 fluorometer using fluorescein as a standard. The pH measurements were carried out on a Corning pH meter equipped with a Sigma-Aldrich micro combination electrode calibrated with standard buffer solutions. Fluorescence images of the live cells under various conditions were acquired via a Zeiss LSM 710 laser scanning confocal microscope.
2. Synthesis and Characterization of CR-Ac (NMR and MS spectra see Figures S9-S16)

Synthesis of CR-Ac

CR was synthesized according to the published methods,\textsuperscript{[1]} and described briefly below.

**Synthesis of 3-acetyl-7-(diethylamino)-2H-chromen-2-one.** A solution of 4-diethylamino-salicylaldehyde (1.93 g, 10 mmol) and ethyl acetoacetate (1.95 g, 15 mmol) in EtOH (15 mL) was treated with piperidine (0.2 mL) and glacial acetic acid (2 drops) and refluxed for 6 h. The mixture was cooled to room temperature and the yellow crystalline solid was filtered. The product was pure enough to be used in the next procedure. $^1$H NMR (CDCl$_3$, 400 MHz $\delta$(ppm)): 8.46 (s, 1H), 7.45 (d, 1H), 6.68 (d, 1H), 6.50 (s, 1H), 3.52 (q, 4H), 2.67 (s, 3H), 1.26 (t, 6H); ESI-MS: found: $m/z = 259.1$ [M+H]$^+$, calcd for C$_{15}$H$_{17}$NO$_3$ = 259.3.

**Synthesis of 2-(7-diethylamino-2-oxo-2H-1-benzopyran-3-yl)-4-(2-carboxyphenyl)-7-diethylamino-1-benzopyrylium (CR).**\textsuperscript{[1]} 1 (0.939 g, 3 mmol) and 2 (0.777 g, 3 mmol) were dissolved in conc. H$_2$SO$_4$ (15 mL) and stirred at 90 °C for 6 h. After cooling to room temperature, the solution was added into ice (30 g) then 70% perchloric acid (1.5 mL) was added, filtered, and washed with water to afford crude product. The crude product was purified by silica gel flash chromatography using CH$_2$Cl$_2$/ CH$_3$OH (50 : 1 to 20 : 1) as eluent to afford
pure CR as a blue solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.33 (s, 1H), 8.22 (d, $J$=8.1, 1H), 8.03-8.05 (m, $J$ = 8.6 Hz, 2H), 7.72 (t, $J$ = 7.4 Hz, 1H), 7.66 (t, $J$ = 7.4 Hz, 1H), 7.52 (Carbon-13 satellite of CHCl$_3$ solvent), 7.50 (s, 1H), 7.34 (d, $J$ = 7.4 Hz, 1H), 7.31 (s, 1H), 7.26 (s, 1H), 7.09 (d, $J$ = 8.3 Hz, 1H), 7.00 (Carbon-13 satellite of CHCl$_3$ solvent), 6.75 (d, $J$ = 8.3 Hz, 1H), 6.46 (s, 1H), 5.30 (s, solvent impurity peak, dichloromethane), 3.67-3.63 (q, $J$ = 7.0 Hz, 4H), 3.53 – 3.48 (q, $J$ = 7.2 Hz, 4H), 1.31 (t, $J$ = 7.0 Hz, 6H), 1.20 (t, $J$ = 7.2 Hz, 6H). ESI-MS: found: $m/z$ = 537.1 [M]$^+$, calcd for C$_{33}$H$_{33}$N$_2$O$_5$ = 537.24 (without ClO$_4^-$)

Synthesis of CR-NH$_2$. CR (0.50 g, 0.07 mmol, 1 equiv) and BOP (1.2 equiv) were dissolved in DCM and stirred at room temperature for 10 min. Then hydrazine monohydrate (0.195 g, 0.35 mmol, 5 equiv) was added. The mixture was stirred at room temperature for 5 h. The solvent was evaporated under reduced vacuum and the crude product was purified by alumina gel column using CH$_2$Cl$_2$ to CH$_2$Cl$_2$/MeOH as eluent to obtain a yellow product (second yellow fraction from the column) (0.130 g). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.36 (s, 1H), 7.91 (d, $J$ = 7.1 Hz, 1H), 7.51 – 7.46 (m, 3H), 7.27 (d, $J$ = 7.4 Hz, 1H), 6.65 (dd, $J$ = 8.9, 2.4 Hz, 1H), 6.50 – 6.49 (s and s, 2H), 6.43 (d, $J$ = 8.8 Hz, 1H), 6.35 (d, $J$ = 8.8, 1H), 6.26 (s, 1H), 3.44 (q, $J$ = 7.2 Hz, 4H), 3.40 – 3.34 (q, 4H, $J$ = 8.4, Hz), 1.25 – 1.18 (m, 12H). $^{13}$C NMR (CDCl$_3$, 100 MHz $\delta$(ppm)): 165.96, 159.50, 156.23, 151.41, 150.75, 148.90, 147.55, 139.71, 132.43, 129.95, 129.93, 128.47, 127.81, 124.11, 123.11, 111.83, 109.46, 109.01, 108.29, 104.18, 100.79, 97.96, 96.72, 64.53, 44.90, 44.33, 13.10, 12.62; ESI-MS: found: $m/z$ = 550.9 [M+H]$^+$, calcd for C$_{33}$H$_{34}$N$_4$O$_4$ = 550.2. The CR-NH$_2$ compound has the same structure as the recently reported CS-ONOO.$^{[2]}$

Synthesis of CR-Ac: CR-NH$_2$ (0.130 g, 0.2 mmol) and acetyl acetone (0.5 ml) were dissolved in ethanol (15 ml). The reaction mixture was then stirred and refluxed for overnight. The solvent was evaporated under reduced vacuum and the crude product was purified by alumina gel column using CH$_2$Cl$_2$ to CH$_2$Cl$_2$/MeOH as eluent to afford a new compound CR-Ac as yellow
solid. (0.043 g, yield ~34%). $^1$H NMR (DMSO-$d_6$, 400 MHz $\delta$ (ppm)): $\delta$ 8.34 (s, 1H), 7.89 (d, $J$ = 6.7 Hz, 1H), 7.49 – 7.44 (m, 3H), 7.26 (d, $J$ = 7.1 Hz, 1H), 6.63 (dd, $J$ = 6.9, 2.4 Hz, 1H), 6.48 and 6.47 (s and s, 2H), 6.42 – 6.39 (d, $J$ = 6.8, Hz, 1H), 6.33-6.31(d, $J$=7.2, 1H), 6.24 (s, 1H), 3.42 (q, $J$ = 7.1 Hz, 4H), 3.33 (q, $J$ = 7.1, 4H), 3.12 (s, 2H), 2.25 (s, 3H), 1.96 (s, 3H), 1.25-1.17 (m, $J$ = 7.2 Hz, 12H). $^{13}$C NMR (CDCl$_3$, 100 MHz $\delta$ (ppm)): 196.4, 165.89, 156.15, 153.50, 151.33, 150.68, 148.82, 147.48, 139.63, 132.35, 129.88, 129.86, 128.39, 127.73, 124.03, 123.03, 111.76, 109.38, 108.93, 108.21, 104.10, 100.72, 97.88, 96.64, 64.53, 44.90, 44.33, 34.31, 12.62, 12.46. ESI-MS: found: m/z = 633.3 [M+H]+, calcd for C$_{38}$H$_{40}$N$_4$O$_5$ = 632.3.

3. Cell Culture and Confocal Imaging

Human primary fibroblast cells ws1 (ATCC, Manassas, VA) was cultured in 25 cm$^2$ culture flasks in Eagle’s Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS), under the condition of 5% carbon dioxide and 37.0°C. The cells were routinely subcultured using 0.25% trypsin, 0.53 mM EDTA solution. The cells were seeded on 35 mm diameter dishes and the medium was changed on the following day. After incubation for 4 hrs, different test materials were added to the medium. Fluorescence imaging was performed with a Zeiss LSM 710 laser scanning confocal microscope. Typically, three experiments were carried out: (1) the cells were incubated with the sensor CR-Ac for 30 min; (2) cells were first incubated with a copper(II) source (CuCl$_2$) and then the medium was removed and replaced with fresh medium containing CR-Ac, or (3) cells were pre-incubated with a copper chelator (SIH). Cell imaging was then carried out after changing cells media. Emission was collected at 460-540 nm for blue channel (excited at 458 nm) and 650-750 nm for red channel (excited at 633 nm). For in situ cell calibration experiments, the cells in the regular medium were subsequently rinsed and replaced with FBS-free EMEM medium, then pyrithione (ATCC, Manassas, VA) was added to each dish to a
final concentration of 7.5 µM. Cells were then incubated with Cu$^{2+}$ at defined concentrations for one hour before confocal images were taken with 10 µM CR-Ac. For images with MitoTracker Green FM, LysoTracker Blue DND-22 and Hoechst 33258, excitation wavelengths were set following the protocols provided by the manufacturer. Emissions were integrated at 492-548 nm (MitoTracker), 409-484 nm (LysoTracker) and 426-535 nm (Hoechst 33258), respectively. The REUSE function controlled by Zeiss software was applied to guarantee that all spectra were recorded under the same instrumental conditions.

4. Supplementary figures (S1-S16)

**Figure S1.** Fluorescence response of 20 µM CR-Ac to various metal ions (20 µM for Cu$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Hg$^{2+}$, Zn$^{2+}$, Ag$^{+}$, Mg$^{2+}$, Pb$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Fe$^{2+}$, Cu$^{+}$ and Cr$^{3+}$; 100 µM for Na$^{+}$, K$^{+}$, Mg$^{2+}$ and Ca$^{2+}$) in ACN/MOPS buffer (10 mM, pH 7.04, v/v 1:1)
**Figure S2.** Variation of absorption (658 nm) of CR-Ac and CR-Ac + Cu$^{2+}$ (20 µM each) at various pH values in ACN/H$_2$O (1/1, v/v) solution.

![Graph showing absorption variation](image)

**Figure S3.** Job's plot. The total concentrations of CR-Ac and Cu$^{2+}$ were kept constant at 40 µM and the absorption intensity was measured at 658 nm in ACN/MOPS buffer (10 mM, pH 7.04, v/v 1:1).

![Job's plot graph](image)

**Figure S4.** In solution linear relationship between the ratio F696/F520 nm and Cu$^{2+}$ concentration (0–10 µM). The limit of detection (DL) of CR-Ac towards Cu$^{2+}$ was determined to be 0.20 µM using a Perkin Elmer LS55 luminescence spectrometer.

![Linear relationship graph](image)
Figure S5. Reversibility of binding of CR-Ac (20 μM) to Cu$^{2+}$ ions. Black line: free CR-Ac (20 μM), blue line: Cr-Ac + 1 equiv of Cu$^{2+}$, red line: CR-Ac + 1 equiv of Cu$^{2+}$ + 5.0 equiv of EDTA.

Figure S6. In situ linear relationship between the ratio F$_{\text{red}}$/F$_{\text{blue}}$ and Cu$^{2+}$ concentration (0–400 nM) in live ws1 cells. Limit of detection (DL) of CR-Ac towards free [Cu$^{2+}$] in cells is determined to be 6.97 nM by LSM710 confocal microscopy using fluorescence ratiometric method.
Figure S7. Representative confocal microscopy images of intracellular colocalization studies of 10 µM CR-Ac incubated with Cu²⁺-loaded ws1 cells (pre-implemented with 50 µM Cu²⁺) co-labeled with LysoTracker Blue (100 nM, incubated for 30 min) (a) CR-Ac-Cu²⁺ fluorescence collected at 650-800 nm (red). (b) LysoTracker fluorescence collected at 409-484 nm (blue). (c) DIC image of (a) and fluorescence images of (b) and (c) were merged together. No colocalization between red and blue images was observed, suggesting that Cu²⁺ is not located in lysosomes.

Figure S8. Representative confocal microscopy images of intracellular colocalization studies of 10 µM CR-Ac incubated with Cu²⁺-loaded ws1 cells (pre-implemented with 50 µM Cu²⁺) co-labeled with MitoTracker Green FM (100 nM, incubated for 30 min). (a) CR-Ac-Cu²⁺ fluorescence collected at 650-800 nm (red). (b) MitoTracker fluorescence collected at 492-548 nm (green). (c) DIC image of (a) and fluorescence images of (b) and (c) were merged together.
Colocalization regions are in orange. A complete colocalization between red and green images was observed, suggesting that Cu$^{2+}$ is located in mitochondria of ws1 cells.
**Figure S9.** ESI-Mass spectrum of CR

**Figure S10.** $^1$H NMR spectrum of CR in CDCl$_3$
Figure S11. ESI-Mass spectrum of CR-NH₂
Figure S12. $^1$H NMR spectrum of CR-NH$_2$. 
**Figure S13.** $^{13}$C NMR spectrum of CR-NH$_2$
Figure S14. Q-TOF Mass spectrum of CR-Ac
Figure S15. $^1$H NMR spectrum of CR-Ac.
Figure S16. $^{13}$C NMR spectrum of CR-Ac.
5. Calculation of Quantum Yield

Stock solutions of 40 μM CR-Ac, 40 μM Rhodamine B (standart), and 40 μM CR-Ac + Cu²⁺ were prepared. Dilutions of CR-AC, Rhodamine B, CR-Ac + Cu²⁺ were prepared in EtO at concentrations such that their absorbance at 658 nm equaled 0.1, 0.2, 0.3, 0.4, and 0.5 μM. Excitation was performed at 650 nm and collected emission was normalized to the EtOH blank and then integrated from 720-800. A plot of the integrated fluorescence intensity vs. the absorbance at 658 nm for each concentration was prepared and the positive slope of the linear fit was calculated. The data were compared to the rhodamine standard using the following equation, where ΦR is the quantum yield of the standard (0.97)[3], Grad is the slope of the absorbance vs. emission line found for each compound, GradR is the slope found for the Rhodamine standard, η is the refractive index of the sample solutions (1.33) and ηR is the refractive index of the rhodamine solution (1.33):

\[
Φ = ΦR \times \frac{\text{Grad}}{\text{GradR}} \times \left( \frac{η^2}{ηR^2} \right) \quad \text{(ΦCR-Ac = 0.02) and (ΦCR-Ac + Cu²⁺ = 0.240)}
\]

6. Calculation of the Binding Constants of the Complex

We estimated the binding constant by using the absorption titration results. The equations below were used to calculate the binding constants with stoichiometry of 1:1..

\[
L + S \rightleftharpoons LS
\]

Where L is the sensor (ligand), S is Cu²⁺ (substrate), and LS is sensor-Cu²⁺ complex.

The apparent binding constant is given by

\[
K = \frac{[LS]}{[L][S]}
\]

Here, the concentrations are at equilibrium.

\[
F_c = \frac{A_m - A_u}{A_u - A_c} = \frac{[LS]_e}{[L]_0}
\]

F_c is the fraction of L that formed a complex, [LS]_e is concentration at equilibrium, [L]_0 is the initial concentration. A_u, A_m, and A_c are the absorbances of solutions of L (before any Cu²⁺ was added), during the titration and at saturation, respectively. The concentration of free Cu²⁺ at equilibrium, [S]_e, is found with the following identity.
\[ [S]_e = [S]_0 - [LS]_e = [S]_0 - F_c [L]_0 \]

The apparent binding constant \( K \) can then be calculated from

\[ K = \frac{F_c}{1-F_c} X \frac{1}{[S]_e} \]

7. References

2. S. Feng, D. Liu, and G. Feng, Analytica Chimica Acta, 2019, 1054, 137-144