Supporting Information

Sensitive and effective imaging of carbon monoxide in living systems with near-infrared fluorescent probe

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1. Materials and Instruments

Benzaldehyde, p-hydroxyacetophenone, diethylamine, nitromethane, ammonium acetate disopropylethylamine, boron trifluoride-diethyl ether, allyl chloroformate, NaOH, Tween-80, PdCl$_2$ and CORM-3 were purchased from Aladdin. Commercial dyes and CCK-8 are from Thermo Fisher Scientific. Unless otherwise stated, all other chemicals are from commercial sources and are analytical reagent grade. All experiments always use ultrapure water.

Performed on silica gel plate (TLC. Hitachi U-2910 for UV absorption spectroscopy experiments. Fluorescence spectroscopy experiments were performed on Hitachi F4600 fluorescence spectrophotometer. $^1$H NMR and $^{13}$C NMR were obtained from BrukerAM 400 MHz and 100 MHz spectrometers, respectively. HR-MS The data is obtained by Agilent 1290 infinity 6540 UHD accurate quality Q-TOF MS (Agilent, USA). The laser scanning confocal microscope (Olympus FV1000) uses a 60-fold objective lens to obtain cell fluorescence images. The images are collected and used Olympus FV10-ASW Ver.2.1 b software for processing. FBS, DMEM and PBS were purchased from Gibco, USA. All kinds of cell line were purchased from the Typical Culture Collection Committee of the Chinese Academy of Sciences (Shanghai, China). The probe (10 mg) was dissolved in deuterated chloroform (0.5 mL) and used for $^1$H NMR, $^{13}$C NMR, HR-MS to characterize its structure.

2. Synthesis and Characterization of the probe

![Figure S1. The general synthetic routes for the probe](image-url)
(1) Synthesis of compound 1

Under argon protection, add benzaldehyde (424 mg, 40 mmol) dropwise to the ethanol solution containing 10% sodium hydroxide and p-hydroxyacetophenone (472 mg, 20 mmol) under ice-water bath, then stir at room temperature 24h. After the reaction is completed, the mixed reaction solution is neutralized with a dilute hydrochloric acid solution, and the pH is adjusted to 6 to obtain a large amount of light yellow powder. After filtration, wash with cold ethanol and water alternately, and dry in vacuum. Compound 1 is a yellow solid, 372 mg. Yield: 83.0%.

(2) Synthesis of compound 2

Add diethylamine (9.2 mL, 90 mmol) and nitromethane (9.6 mL, 180 mmol) to the stirred ethanol solution of compound 1 (404 mg, 18 mmol), and reflux the mixture in ethanol (50 mL) for 24h. After cooling down to room temperature, add dilute hydrochloric acid again to adjust the pH to 6. Subsequently, the reactant was extracted with ethyl acetate and saturated brine, the organic layer was collected, and 395 mg of a brown solid compound was obtained after rotary evaporation. Yield: 77.0%.

(3) Synthesis of compound 3

Compound 2 (200 mg, 7.0 mmol) and ammonium acetate (189 mg, 245 mmol) were added to ethanol (50 mL) and heated to reflux for 48 h. The mixed solution was cooled to room temperature, the reaction solution was extracted with ethyl acetate and saturated brine, and the organic phase was collected, dried over anhydrous sodium sulfate and rotary evaporated to obtain 123 mg of a blue-black solid compound. Yield: 73.1%.

(4) Synthesis of compound 4

Under an argon atmosphere, compound 3 was dissolved in dry dichloromethane (30 mL), and then diisopropylethylamine (0.54 mL, 3.11 mmol) and boron trifluoride-diethyl ether (0.55 mL, 4.35 mmol) and stirred at room temperature for 24h. After completion, the reaction mixture was extracted in dichloromethane (50 mL) and saturated brine (50 mL), and the organic layer was collected and evaporated to dryness. Finally, the resulting product was purified by column chromatography with dichloromethane/ethyl acetate (4:1 v/v) as the eluent, and 7 mg of metallic red solid compound was obtained. Yield: 42.7%.

(5) Synthesis of compound 5

Under an Ar atmosphere, compound 4 (99.7 mg, 0.1 mmol) and triethylamine (30 μL, 0.21 mmol) were added to a round bottom flask containing 50 mL of dichloromethane. The allyl chloroformate was slowly added (16.3 μL, 0.2 mmol) into the mixed solution at 0 °C, and then further stirred at 25 °C for 6 h. After the solvent was removed under reduced pressure, the obtained product was purified by silica gel chromatography (200 - 300 mesh) with gradient eluents CH₂Cl₂ and CH₃OH (100:1 to 5:1, v:v). The target compound 5 (39.91 mg, yield: 38%) was obtained. ¹H NMR (400 MHz, CDCl₃) δ (ppm): 8.09-8.02 (m, 7H), 7.46-7.41 (m, 6H), 7.34-7.31 (d, 4H), 7.00 (s, 2H), 6.06-5.95 (m, 2H), 5.47-5.43 (dd, 2H), 5.36-5.34 (dd, 2H), 4.77-4.76 (d, 4H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 158.35, 152.99, 152.92, 145.66, 144.34,
3. Spectral experiment

The absorption and fluorescence spectra were measured in a 10 mM HEPES (pH 7.4, 37°C) solution. Purge HEPES with nitrogen for 5 min before use. By adding different volumes of HCl or NaOH solutions, the pH gradient of the HEPES buffer solution is 3.0 ~ 10.0. Prepare probe (1 mM) and PdCl$_2$ (10 mM) stock solutions in HPLC grade DMSO, respectively. A stock solution of tricarbonyl chloride (glycine) ruthenium(II) (CORM-3, a CO releasing molecule that can safely release CO) was prepared in HPLC grade DMSO and used fresh. In the ultrapure water prepared by the Millipore-Q ultra-purification system, stock solutions of other analytes including amino acids, metal anions and cations were prepared. Reactive oxygen/nitrogen species (ROS/RNS) were prepared according to the procedures previously published in this article, and has been freshly used. Dilute the probe and PdCl$_2$ to 10 µM in HEPES buffer solution (pH 7.4, 0.5% DMSO, 0.5% Tween80). Put 3.0 mL of the probe solution into a quartz cuvette, and control it at 37°C by a temperature controller. After the mixture is equilibrated for 5 minutes, it is ready for determination. After adding different concentrations of CORM-3, then record the UV-Vis or fluorescence spectra. The UV absorption spectrum was measured on the NanoDrop 2000/2000C UV-Vis spectrophotometer. Fluoromax-4 fluorescence spectrophotometer is used to carry out the fluorescence spectroscopy experiment of the probe, which is equipped with a xenon lamp and a 1.0 cm quartz cuvette. $\lambda_{ex/em}$ = 690/710-850 nm.

4. Cell Culture and confocal imaging

Fluorescence images were collected on the Olympus FV1000 confocal laser scanning microscope. The cells were plated in a confocal petri dish and cultured adherently for 24 h. Before imaging, rinse the confocal petri dish three times with 1 mL PBS, and then the probe (10 µL, 1.0 mM) was added. After different treatments, the cells were washed three times with PBS, and then the cells were imaged. First inoculate live cells on a confocal plate, then add 1 ml of fresh complete medium, and then culture for 24 h. Before performing the imaging experiment, the cells were incubated with 10 µM probe and 10 µM PdCl$_2$. Confocal images of cells were obtained using Olympus FV-1000 laser scanning microscopy and 60× oil objective lens. Green channel: the excitation wavelength was selected as 690 nm, and the collection wavelength was selected as 710-850 nm.
5. Comparison of the properties of fluorescent probes for CO

| Probe | Emission wavelength | Pd-free | LOD  | Linear range | Ref  |
|-------|---------------------|---------|------|--------------|------|
| ![ Probe 1 ] | 445 nm | Yes | 12 nM | 0-20 μM | 30 |
| ![ Probe 2 ] | 560 nm | NO | 50 nM | 0-1.5 μM | 27 |
| ![ Probe 3 ] | 520 nm | NO | 46 nM | 0-50 μM | 29 |
| ![ Probe 4 ] | 415 nm | NO | 60 nM | 0-6 μM | 28 |
| ![ Probe 5 ] | 515 nm | Yes | Not mentioned | 0-10 μM | 31 |
| ![ Probe 6 ] | 748 nm | NO | 45 nM | 0-200 μM | This work |

Table S1. Comparison of the properties of fluorescent probes for CO

6. Concentration ratio of the probe to PdCl$_2$

As shown in Figure S2, although increasing the amount of PdCl$_2$ was found more favorable for fluorescence signal changes, fluorescence enhancement becomes slower after addition of one equivalent of PdCl$_2$. Thus, a 1:1 ratio of the probe and PdCl$_2$ was used in the following studies because this ratio is good enough for detection of CO with a remarkable fluorescent readout.
**Figure S2.** Fluorescence intensity changes of the probe (10 μM) for CORM-3 (200 μM) with different concentrations of PdCl₂ (0-25 μM) in HEPES buffer solution (pH 7.4, 0.5% DMSO, 0.5% Tween-80 and λ_{ex}=690 nm) at 37°C. Each data was recorded 5 min after mixing.
7. The effect of Tween-80 on the fluorescence intensity of the probe

![Graph showing the effect of Tween-80 on fluorescence intensity.](image1)

**Figure S3.** The effect of Tween80 on the fluorescence intensity of the probe (10 μM, 0.5% DMSO). The data was obtained under the conditions of 10 mmol/L HEPES (pH 7.4, 0.5% DMSO). (λ<sub>ex</sub>/λ<sub>em</sub> = 690/710-850 nm)

8. Linear relationship between probe fluorescence intensity and CORM-3 concentration

![Graph showing the linear relationship between probe fluorescence intensity and CORM-3 concentration.](image2)

**Figure S4.** The linear relationship between probe fluorescence intensity (610 nm) and CORM-3 concentration (0-200 μM). The experiment is repeated three times, and the data is averaged (± s.d.)
9. The selectivity of the probe to other analytes

**Figure S5.** Fluorescence intensity of the probe response to various analytes in HEPE. (a) 1 Nature; 2 Gln(15 μM); 3 Thr(15 μM); 4 Glu(15 μM); 5 Ser(15 μM); 6 Asp(15 μM); 7 Leu(15 μM); 8 Ile(15 μM); 9 Arg(15 μM); 10 Met(15 μM); 11 Tyr(15 μM); 12 Trp(15 μM). (b) 1 K⁺(1 mM); 2 Na⁺(1 mM); 3 Ca²⁺(1 mM); 4 Mg²⁺(1 mM); 5 Zn²⁺(1 mM); 6 Cu²⁺(1 mM); 7 Cl⁻(1 mM); 8 Br⁻(1 mM); 9 Fe²⁺(1 mM); 10 Fe³⁺(1 mM); 11 Ca²⁺(1 mM); 12 Ag⁺(1 mM). (c) 1 HNO(200 μM); 2 H₂PO₄⁻(200 μM); 3 SO₄²⁻(200 μM); 4 CO₃²⁻(200 μM); 5 HCO₃⁻(200 μM); 6 ClO⁻(200 μM); 7 OH⁻(200 μM); 8 ROO⁻(200 μM); 9 ONOO⁻(200 μM); 10 H₂O₂(200 μM); 11 CO₃²⁻(200 μM); 12 CORM-3(200 μM). The data was obtained under the conditions of 10 mmol/L HEPES (pH 7.4, 0.5% DMSO and 0.5% Tween 80), the excitation wavelength was 690 nm, and the emission wavelength was 745 nm. The experiment was repeated three times, and the data was averaged (±s.d.).

10. Cytotoxicity assays

FaDu cells were placed in MEM with 10 % FBS, HeLa, PC12, RAW 264.7 and A549 cells were placed in DMEM with 10 % FBS, cultured at 37°C in 5 % CO₂ and 95 % air. Five kinds of cells (8000 cells/well) were respectively seeded into 96-well plates, and adherent culture for 24h. Subsequently, the cells were incubated with 0.001, 10, 20, 30, 40, 50, 60, 70, 80 and 100 µM (final concentration) probes (dissolved in DMSO) at 37°C in 5 % CO₂ and 95 % air. At 24h, under the same conditions as the control, untreated DMEM was also tested. Add CCK-8 solution (5.0 mg/mL, 10 µL) to each well. Then the plate was incubated in 5 % CO₂ and 95 % air for 1 h, and then the absorbance was measured at 750 nm using TECAN infinite M200pro.
Figure S6. The effect of different concentrations of probes on cell viability. (a) RAW264.7; (b) FaDu; (c) A549; (d) PC12; (e) HeLa. The experiment was repeated three times, and the data was averaged (±s.d.).

11. Preparation of Cell Hypoxia Model
Inoculate RAW 264.7 cells at a density of $10^5$/ml in 96-well plates (100 µL/well), and according to hypoxia time 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h were randomly divided into 11 groups. After the cells are fully attached, the hypoxic group cells are placed in a transparent glass box, and then the glass box is placed in a 37 °C incubator for culture, and then pass 5 % CO$_2$, 1 % O$_2$ and 94 % N$_2$ gas cylinders into the glass box, set the gas outlet, and continue to mix the gas. The hypoxia treatment was 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h. The normal experimental group was continuously cultured in an ordinary incubator containing 5 % CO$_2$ at 37 °C.

12. Bright-field cell images of Figure 3
Figure S7. Bright-field cell images of Figure 3.

13. Bright-field cell images of Figure 4

Figure S8. Bright-field cell images of Figure 4.

14. $^1$H NMR, $^{13}$C NMR and HR-MS of the probe.
Figure S9. $^1$H NMR of the probe

Figure S10. $^{13}$C NMR of the probe
Figure S11. HR-MS of the probe