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Communication

Palladium-free chemoselective probe for *in vivo* fluorescence imaging of carbon monoxide

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ABSTRACT

Carbon monoxide (CO) is a vital intracellular gas messenger known for its cytoprotective and homeostatic properties. It plays a pivotal role in a myriad of biological processes. Therefore, the precise detection of CO is of paramount importance in unraveling the intricacies of pathological mechanisms and advancing the development of disease diagnosis. We herein introduce NFCOP, a state-of-the-art near-infrared (NIR) turn-on fluorescence (FL) probe that has been meticulously designed for highly sensitive, swift and selective imaging of CO. The NFCOP response occurred rapidly with CO, within just 10 s, and the calculated detection limit for CO was determined to be 0.32 μmol/L. Further investigations conducted at the cellular level and *in vivo* demonstrated that NFCOP possesses high sensitivity and selectivity for imaging CO.

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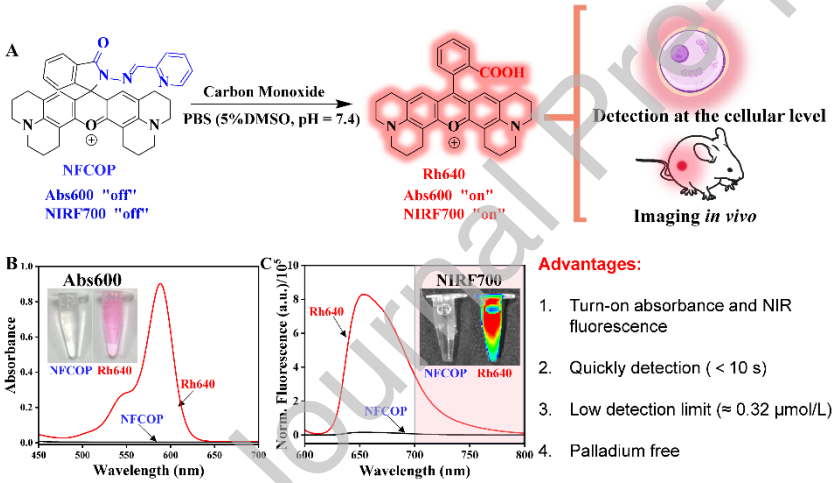
Carbon monoxide (CO) is recognized as a crucial gas-signaling molecule generated endogenously through theme breakdown. It plays a significant role in regulating various aspects of animal physiology [1-3]. Due to its involvement in serious diseases such as hypertension, inflammation, cancer, and cell apoptosis, as well as its potential as a therapeutic drug, CO has become a subject of growing interest [4-6]. As a result, the accurate and reliable detection of CO at both the cellular and *in vivo* levels is of paramount importance. Such detection methods are essential for advancing our understanding of pathological mechanisms and improving the accuracy of disease diagnosis [7,8].

Traditional methods for CO detection predominantly rely on electrochemical analysis [9,10], coordination chemical colorimetry [11], and gas chromatography [12,13]. However, these conventional approaches exhibit limitations render them unsuitable for the real-time monitoring of endogenous CO within biological samples [14]. By comparison, fluorescence (FL)-based detection methods have garnered dramatic attention in the realm of biomedical research due to their remarkable attributes, including heightened sensitivity, exceptional temporal resolution, and rapid data acquisition [15,16]. Up to now, many efforts have been made in the development of fluorescent probes for endogenous CO, and great progress has been obtained. For example, Chang's group reported a new type of turn-on fluorescent probe for selective CO detection based on palladium-mediated carbonylation reactivity, which exhibits the remarkable capability of CO detection in diverse environments, including aqueous buffer solutions and live cellular systems [17]. However, several challenges remain to be addressed. As shown in Table S1 (Supporting information), the characteristics of previous CO FL probes have been summarized. Firstly, the detection time required by the majority of probes exceeds 5 min and even up to 60 min. This extended detection time poses a significant drawback, particularly when real-time or rapid detection is required in various applications. Secondly, the reliance on auxiliary reagent palladium (Pd) for the activation of numerous probes hampers their extensive application *in vivo*, considering the toxicity associated with heavy metal ions. Thirdly, metal-free fluorescent probes predominantly exhibit excitation and emission wavelengths in the blue and green light spectrum, lacking the capability for near-infrared region fluorescence (NIRF), which limits the tissue penetration for deep tissue imaging, because NIRF (700–2500 nm) can penetrate biological tissues more efficiently than visible light [18-20]. The above considerations inspire us to further develop better FL probes to explore the biological functions of CO.

In this study, we presented the synthesis and application of NFCOP, a novel NIRF probe engineered for the swift, sensitive, and selective detection of CO in both *in vitro* and *in vivo* settings. The NFCOP is designed with a rhodamine 640 perchlorate scaffold and incorporates a 2-(hydrazonomethyl) pyridine moiety in a lactam form as the CO-reactive group (Scheme 1A). Harnessing the potential of NIRF imaging techniques, our study underscores the exceptional sensitivity and selectivity of the NFCOP probe for CO detection, both at the cellular level and in *in vivo* scenarios.



Scheme 1. (A) Illustration of NIRF turn-on response of NFCOP for CO detection. (B) Absorbance and (C) fluorescence emission spectra of NFCOP (24 μmol/L) after treatment with CO (the concentration of CORM-3: 24 μmol/L, dissolved in the 5% DMSO of water, pH 7.4, reaction time: 30 s).

Based on the rhodamine 640 perchlorate framework, NFCOP was synthesized *via* the incorporation of 2-(hydrazonomethyl) pyridine as a CO-reactive moiety in a lactam configuration. The synthesis process is outlined in Fig. S1 (Supporting information). Initially, the pivotal intermediate of rhodamine 640 perchlorate hydrazide was synthesized. Subsequently, the free amino group was subjected to an addition-condensation reaction with 2-pyridine carboxaldehyde, resulting in the formation of the pyridine hydrazone compound NFCOP. The structure of the obtained probe was meticulously characterized through the utilization of techniques such as ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectral analyses (Figs. S2–S7 in Supporting information). The photophysical properties of NFCOP were measured in aqueous solution (5% DMSO, pH 7.4) at 37 °C. Referring to previous work, tricarbonylchloro(glycinato)ruthenium(II) (CORM-3), as a water-soluble CO-releasing molecule, is used as a CO donor in this experiment. As shown in Figs. S8 and S9 (Supporting information), the results indicate that each mole of CORM-3 can liberate ≈0.77 mol of CO in solution [21,22]. When exposed to CO-containing solutions, NFCOP could be effectively hydrolyzed by CO, resulting in an absorption peak turn-on and strong fluorescence emission (Fig. S10 in Supporting information). The change in optical properties of NFCOP after CO interaction is attributed to the hydrolysis reaction caused by the formation of unstable intermediates [23,24]. To obtain more accurate experimental results, the distribution of CO in cells and *in vivo* could be imaged by using the NIR700 (FL at 700 nm) of the NFCOP probe as an indicator in subsequent experiments.

To demonstrate the responsiveness of NFCOP to CO, the absorption spectral changes of NFCOP toward CO were analyzed using ultraviolet and visible (UV–vis) spectrophotometry. As illustrated in Fig. 1A, with the introduction of CORM-3, the absorbance of NFCOP (0–24 μmol/L) increased gradually. The results from electrospray ionization mass spectrometry (ESI-MS) analysis indicated that the predominant component of the product was primarily rhodamine 640, which verified that NFCOP interacted with CO through a hydrolysis reaction (Fig. S11 in Supporting information). Furthermore, a linear correlation of the Abs600 and the CO concentration in the range of 24 μmol/L was observed (R^2 = 0.988). The fluorescence signal of NFCOP was observed upon exposure to varying concentrations of CO. As depicted in Fig. 1B, initially, there was no detectable NIRF 700 from NFCOP. After the reaction with CORM-3 for 30 s, when the concentration of NFCOP reached saturation at 24 μmol/L, the NIRF700 intensity exhibited an increase of 52.3-fold compared to the initial baseline. Notably, a strong linear correlation was observed between NIRF700 intensity and CO concentration (0.32 μmol/L) (R^2 = 0.998). The aforementioned results provided strong evidence supporting the exceptional selectivity and sensitivity of probe NFCOP in detecting CO. The design of CO chemoselective probes mainly relied on the Pd^0-mediated TsujiTrost reaction and the CO-triggered clearance of the cyclo-Pd complex reaction before, while Pd-free probe is an important expectation in design of NFCOP, as shown in Fig. 1C, NFCOP completes the process of responding to CO without Pd, the presence or absence of Pd make no difference to the experimental results of the response of NFCOP to CO. Furthermore, an investigation into the reaction kinetics between NFCOP and CO was conducted. To our surprise, without the involvement of any metal auxiliary agents, after different concentrations of CO (4, 12, 24 μmol/L) were added into the NFCOP solution, the NIR700 intensity exhibited a gradual increase over time, eventually plateauing within a span of 10 s. (Fig. 1D). The effect of the pH of the solution under physiological conditions on NFCOP detection of CO can be almost negligible (Fig. S12 in Supporting information). There is a similar recognition effect for free gaseous CO (Fig. S13 in Supporting information). To the best of our knowledge, it is also the lowest level in the previously published work about optical probes of CO detection in the NIR spectral range, indicating the advantage of rapid response compared to previous probes of NFCOP (Table S1 in Supporting information).

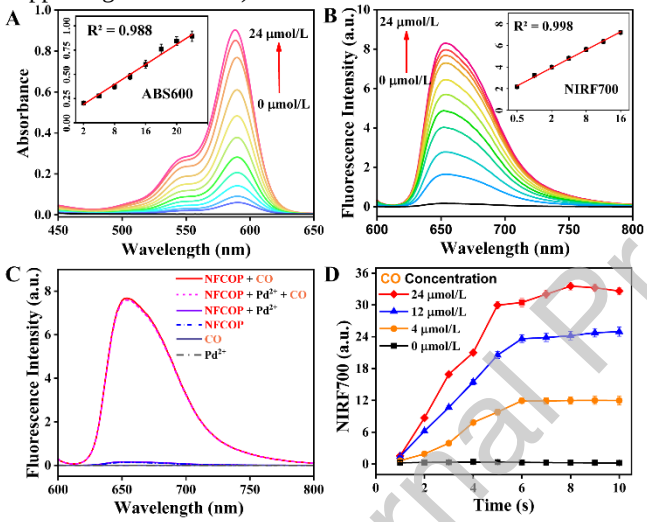


Fig. 1. (A) Fluorescence spectra of NFCOP after different treatments. (B) The Plot of NIRF intensity at 700 nm of NFCOP treated with different concentrations of CO. (C) The UV–vis absorption spectra and (D) FL spectra of NFCOP solutions with different CO concentrations.

Next, the selectivity of NFCOP was also investigated against other various biologically relevant species including Na^+, SO_4^{2-}, Ca^{2+}, Br^-, K^+, Cl^-, NH_4^+, $H_2PO_4^-$, CO_3^{2-}, NO_3^-, H_2O_2, glucose, and urea. The Abs600 intensity demonstrated a turn-on response when exposed to CO (Fig. 2A); however, other species produced no effect or a negative effect. As it is well-established, the mentioned species inherently lack FL signals, and when subjected to incubation with NFCOP, their NIRF700 intensity remained unaltered, with the sole exception of CO (Fig. 2B). This observation underscores NFCOP's excellent selectivity towards CO. Photographic images also demonstrated that NPCOF is in response to CO, changing their colors from colorless to pink (Fig. 2C). More interestingly, NIR images showed a high selectivity of NPCOF against CO (Fig. 2D). These findings imply that NFCOP has the potential to function as a responsive NIR FL probe for the specific detection of CO.

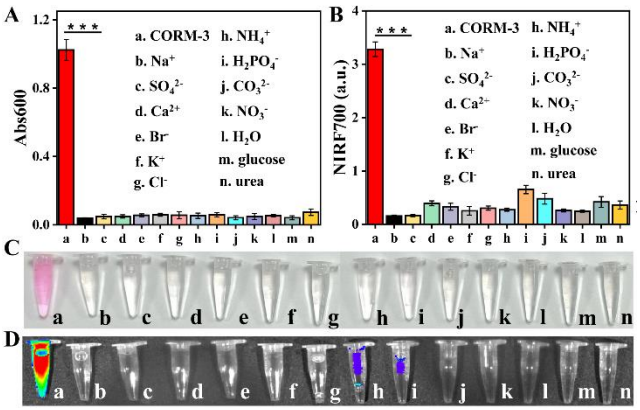


Fig. 2. (A) The Abs600 and (B) NIRF700 of NFCOP in the presence of different analytes (mean ± SD, $n = 3$). ***$P < 0.0005$. (C) Photograph of NFCOP before and after reaction with different analytes. (D) NIR images of NFCOP in the presence of different analytes (a. CORM-3, b. Na^+, c. SO_4^{2-}, d. Ca^{2+}, e. Br^-, f. K^+, g. Cl^-, h. NH_4^+, i. $H_2PO_4^-$, j. CO_3^{2-}, k. NO_3^-, l. H_2O_2, m. glucose, n. urea).

To evaluate the practicability of the NFCOP probe in living organisms, we conducted CO monitoring within Panc02 cells (mouse pancreatic cancer cell line) and L02 cells (human fetal hepatocyte line) using high-resolution confocal imaging. Initially, the potential cytotoxicity of NFCOP was assessed using the cell-counting-kit-8 (CCK-8) assay. Subsequently, Panc02 cells were exposed to varying concentrations of NFCOP (ranging from 0 to 100 μmol/L) for 24 h, and it was determined that over 80% of the cells remained viable (Fig. S14 in Supporting information). These outcomes indicated that NFCOP exhibited negligible cytotoxic effects, even when used at elevated probe concentrations (up to 100 μmol/L). Following this, Panc02 cells were initially preincubated with CO (24 μmol/L) for 4 h, followed by the addition of NFCOP (24 μmol/L) for an additional 1 h incubation period. As shown in Fig. 3A, in the absence of CO, unreacted NFCOP exhibited a negligible NIRF700 signal. However, a substantial NIRF700 signal was observed after the incubation with both CO and NFCOP. In comparison to free NFCOP, the NIRF700 signal generated by NFCOP exhibited a 13.1-fold increase following incubation with CO (Fig. 3B). These findings proved that NFCOP effectively detects CO within living cells by enhancing the NIRF700 signal. What is more, the confocal imaging experiments of co-incubation of green fluorescent lysosome tracker and Panc02 cells showed that NFCOP may be located in lysosome at the cell level. This conclusion is further confirmed by the analysis of the intensity scatter plot of the red-green channels (Fig. 3C).

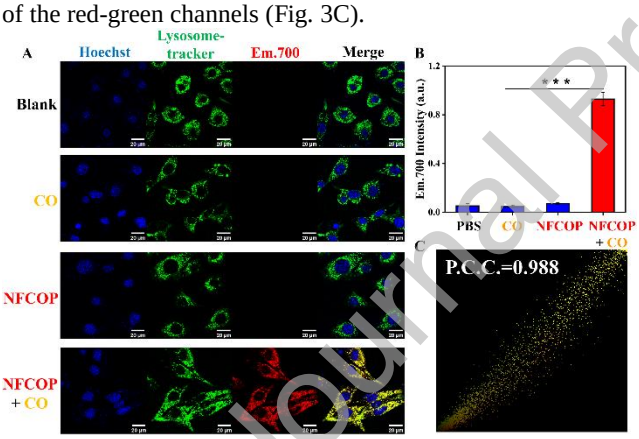


Fig. 3. (A) Confocal fluorescence images of CO in Panc02 cell by the treatment of NFCOP, scale bar: 20 μm. (B) NIRF700 intensity of the red channel (mean ± SD, $n = 3$). ***$P < 0.0005$. (C) Intensity scatter plot of green and red channels.

To further confirm the viability of NFCOP for monitoring CO in live specimens, we employed NFCOP to visualize CO within chicken tissue and subcutaneous mouse tissue. All animal experiments were performed under the protocols approved by the Animal Care and Use Committee (Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences) (Serial number: SIAT-IACUC-210701-YYS-GP-A1974). As illustrated in Fig. 4A, the NIRF700 signal remains visible even after placing a 7 mm-thick layer of chicken tissue on top of the CO-activated NFCOP solution in phosphate buffered saline (PBS), highlighting its exceptional tissue penetration capability. In contrast, the fluorescence signal of rhodamine B vanishes when tissue with a thickness of 2 mm covers it. *In vivo* animal model experiment, the control groups pretreated with CO (24 μmol/L, 100 μL) or NFCOP (24 mol/L, 100 μL) respectively observed negligible NIRF700 signals (Fig. 4B). While the group with the co-existence of CO and NFCOP could ultimately obtain a gradually enhanced NIRF700 signal, which reaches its maximum value at 10 s. Utilizing fluorescence intensity analysis of NIR images, it was observed that in comparison to groups treated solely with NFCOP or CO, the intensity of the NIRF700 signal exhibited a substantial 29.3-fold enhancement in subcutaneous tissue treated with both NFCOP and CO (Fig. 4C). These results suggest that NFCOP can effectively detect CO *in vivo* by significantly enhancing the NIRF700 signal.

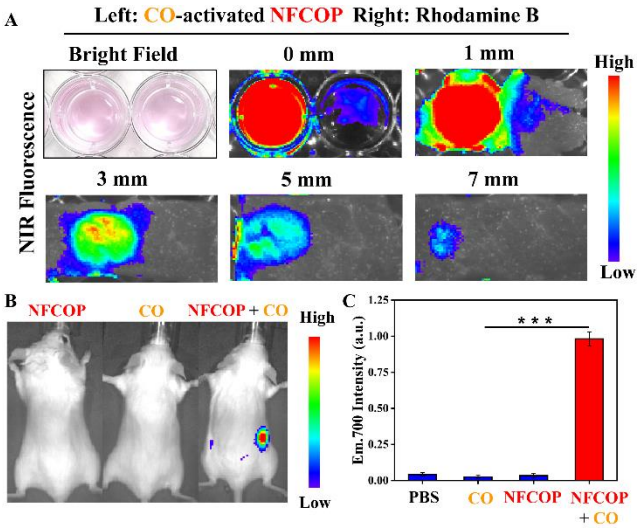


Fig. 4. (A) NIR imaging was performed at 37 ºC with different thicknesses of chicken tissue overlay (CO-activated NFCOP on the left, rhodamine B on the right). (B) Representative NIRF700 images (λ_{ex} = 600 nm). (C) The corresponding NIRF700 intensity changes of mice toward different treatments (mean ± SD, n = 3). ***P < 0.0005.

To assess the *in vivo* toxicity of NFCOP, a histological analysis using hematoxylin and eosin (H&E) staining was conducted on major organs (including the heart, kidney, liver, lung, and spleen) from mice subjected to various treatments. The results, performed 24 h post-injection, as depicted in Fig. S15 (Supporting information), revealed no apparent signs of organ damage or inflammation, suggesting good biocompatibility of NFCOP. Furthermore, the minimal toxicity was further corroborated through the absence of blood histopathological abnormalities or lesions, as supported by sound biochemical and blood routine results. The data from all treatment groups fell well within the normal range (Fig. S16 in Supporting information).

In summary, our study focused on developing a fluorescence probe, NFCOP, that can selectively and sensitively image CO. This palladium-free probe exhibits a remarkable near-infrared (NIR) fluorescence turn-on response to extremely low concentrations of CO, allowing for rapid detection within 10 s. The *in vitro* calculated fluorescence detection limit for CO was approximately 0.32 μmol/L. By leveraging the deep tissue penetration capabilities of NIR imaging and NFCOP's exceptional chemoselective performance, we were able to visualize CO in the subcutaneous tissues of mice successfully. Our findings highlight the significant potential of chemoselective NIR probes for *in vivo* CO imaging applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated to this article can be found, in the online version, at doi:.

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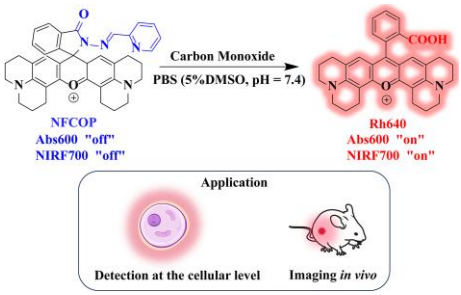
Graphical Abstract



A palladium-free chemoselective probe (NFCOP) that exhibits remarkable near-infrared (NIR) fluorescence turn-on responses toward carbon monoxide (CO) was developed. This response occurred rapidly, within just 10 s, and the calculated detection limit for CO was determined to be 0.32 μmol/L. Further investigations conducted at the cellular level and *in vivo* demonstrated that NFCOP possesses high sensitivity and selectivity for imaging CO.

Declaration of interests

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