Turn-on and Turn-off Fluorescent Probes for Carbon Monoxide Detection and Blood Carboxyhemoglobin Determination

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* Supporting Information

ABSTRACT: Water-soluble, carbazole-based two-photon excitable fluorescent probes MPVC-I ("turn-on") and MPVC-II ("turn-off") are rationally designed and synthesized for the selective monitoring of carbon monoxide (CO). Both probes can effectively measure carboxyhemoglobin (HbCO) in the blood of the animals exposed to a CO dose as low as 100 ppm for 10 min. The palladium catalyzed azidocarbonylation reaction was optimized to improve the sensing efficiency.
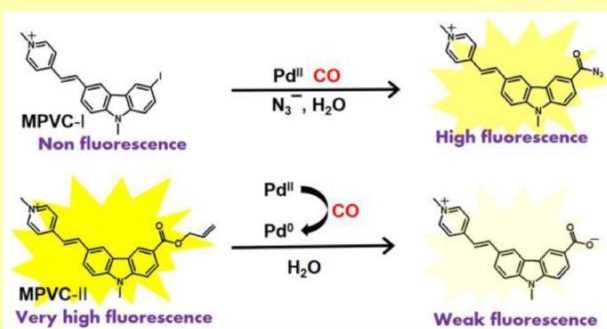
KEYWORDS: fluorescent probe, carbon monoxide, blood carboxyhemoglobin, gas sensing, azidocarbonylation

Carbon monoxide (CO) is a widely applied fuel and a reducing gas in modern industry, but it has a somewhat notorious reputation because of its inhalation toxicity. Uninten-tional and non-fire-related CO poisoning has cost numerous lives.[1,2] According to the World Health Organization (WHO), any exposure to ambient air with CO levels greater than 100 ppm is dangerous to human health[3] because CO possesses a much higher affinity toward the "oxygen transporter" hemoglobin (Hb) than O_2 does. As a result, measuring the carboxyhemoglobin (HbCO) level in blood is of crucial importance in CO-poisoning diagnosis and forensic post-mortem examination.

Solid-material-supported and ligand-exchange-based chromogenic/fluorogenic CO probes with Ru(II),[4−6] Rh(II),[7−9] and Os(II)[10] coordination centers were found to show high selectivities and low detection limits; however, they suffer from poor color modulations toward different CO concen-tration levels and were not tested for the sensitivity toward CO in the presence of other interfering gas species. Fluores-cence,[11−14] including palladacycle-based two-photon fluores-cence,[15−17] and Raman-based[18] CO imaging in cells or tissues have been reported. CO monitoring for bioimaging was also studied via the reduction of Pd(II) to Pd(0), followed by the in situ generated Pd(0) catalyzed Tsuji−Trost reaction.[19−27] However, these CO bioimaging experiments were all conducted using carbon-monoxide-releasing molecules (CORM-2 or

CORM-3) instead of gaseous CO. To the best of our knowledge, there is only one solution-based fluorescence probe for on-site CO gas sensing, which utilizes the azidocarbonylation reactions of aryl iodide,[28] but it requires a relatively high loading of the probe and a long response time. Furthermore, none of the aforementioned methods can be used to carry out the HbCO test.

Modern spectrophotometric, chromate-graphic and electro-chemical techniques[29,30] can perform HbCO tests, but they require delicate instrumentation and tedious, time-consuming pretreatment (especially chromatographic measurements) of whole blood samples. Because there is no simple and quick HbCO detection method, we here report such a method using fluorescence for the first time.

Palladium-catalyzed carbonylation reactions of aryl halides in the presence of CO have been well established; unfortunately, most of these reactions proceed at a relatively high temperature and CO pressure, thus limiting their application to fast-responding CO sensing.[31] Grushin and co-workers reported systematic studies on aromatic azidocarbonylation (carbon-ylative azidation) using azide as a nucleophile and found that

azidocarbonylation could proceed rapidly at ambient temper-ature and pressure;[32,33] moreover, the stable acyl azide intermediates were isolated and characterized by single crystallography. Curtius rearrangements usually do not proceed rapidly in the absence of ortho-substituents on aryl iodide,[34] so it is plausible to suspend the azidocarbonylation reaction at a high fluorescent acyl azide stage within the CO detection time scale.

Herein, having overcome the aforementioned limitations, we disclose our designed two photon excitable[35] and synthetically feasible N-methyl-4-pyridiniumvinylcarbazole based MPVC-I and MPVC-II as "turn-on" and "turn-off" fluorescent CO probes that utilize solution-phase irreversible azidocarbonyla-tion[36] and Truji-Trost reaction (Figure 1), respectively.
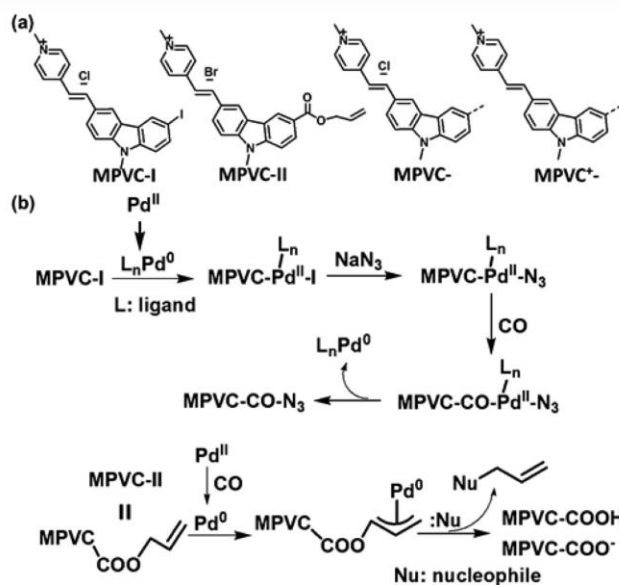

Figure 1. (a) Probes MPVC-I and MPVC-II and substituents MPVC-and MPVC[+]-. (b) Proposed reaction processes of CO sensing using MPVC-I and MPVC-II.

Moreover, we studied the reaction kinetics, which enabled us to optimize the chemical reaction conditions and achieve the best sensitivity for gaseous CO sensing. We also applied our probes for the rapid determination of blood HbCO levels synchronously with CO liberation from the whole blood without any pretreatment to a satisfactory extent.

■ EXPERIMENTAL SECTION

All common reagents and solvents were purchased and used without further purification. New compounds were synthesized and charac-terized by ^{1}H and ^{13}C NMR (400 MHz FT-NMR Model: 400-MR DD2Mag. Res. System at 298 K) and low-resolution mass spectrometry (Bruker 1200 Series and HCT Basic System). Known compounds were characterized by ^{1}H NMR. Fluorescence studies were carried out using a Shimadzu RF-5301 PC Spectrofluoropho-tometer at 298 K. UV−vis spectra were obtained using a Scinco S-3100 Photodiode Array UV−vis Spectrophotometer at 298 K. Liquid chromatography was performed on a C18 column.

Synthesis. We synthesized new compounds according to the synthetic scheme shown in Figure 2 (see Supporting Information (SI) for the details).

Fluorescence Studies. Spectrofluorometric studies were per-formed as follows. Stock solutions of compound MPVC-I (10 mM), MPVC-II (10 mM, washed by acetone, but not purified by acetonitrile, which results in higher yield), sodium azide (1 M), and
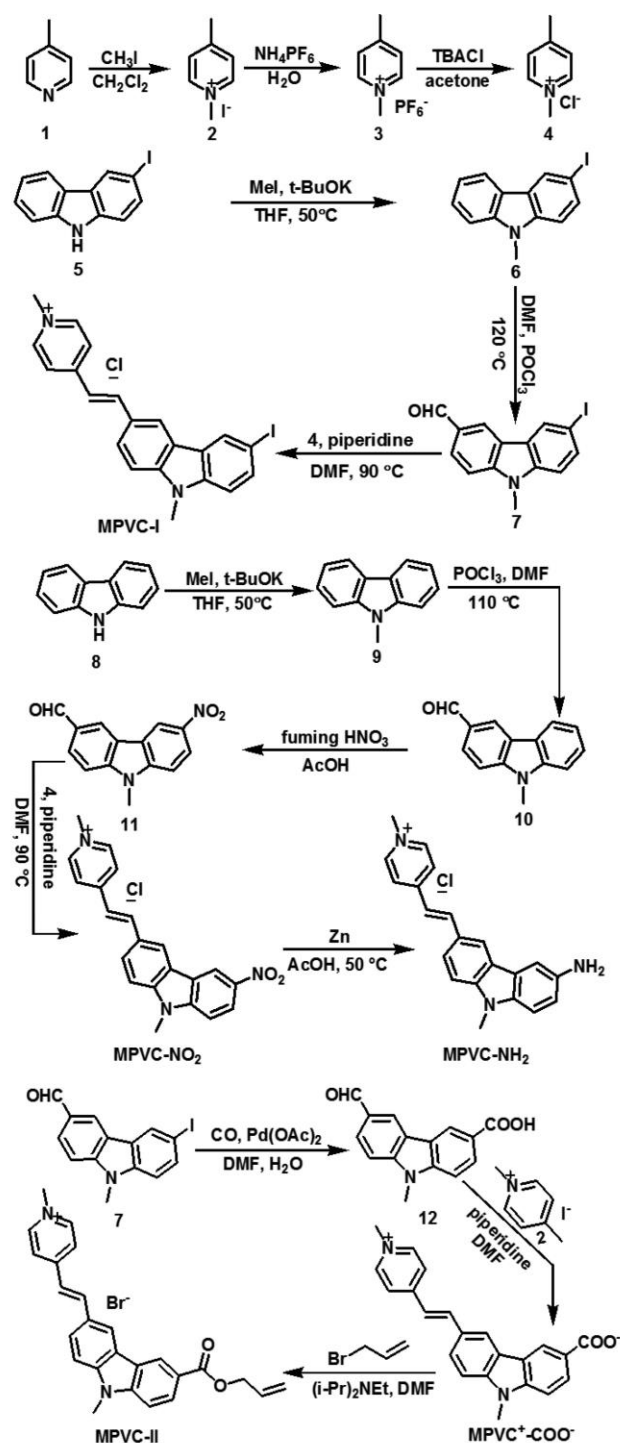

Figure 2. Synthetic scheme of MPVC-I, MPVC-II, and MPVC-NH$_2$ for fluorescence comparison.

each additive (0.2 M ∼ 1 M) were prepared in water. Stock solutions of Pd(OAc)$_2$ (10 mM) and phosphine ligands (5 mM ∼ 10 mM) were prepared in DMSO. PdCl$_2$ (10 mM) was prepared in 100 mM NaCl$_{(aq)}$. Solid sodium acetate (1 mM) was directly dissolved into PBS (10 mM phosphates, pH 7.4). The species for selectivity screening (10 or 100 mM) were prepared in water, except for NaCN in PBS.

Generally, certain volumes of ingredients from each stock solution were added to PBS to reach the specified concentration, and the resulting solutions were vigorously shaken after bubbling CO or adding other analytes. Then, the solutions were kept still for a certain time before scanning (optical path length = 10 mm). The emission spectrum was measured by keeping the slit width at 3 nm. For kinetic

studies of MPVC-I, the samples were kept inside a fluorescence cuvette, and time-dependent scanning was programmed; scanning was stopped after the change in fluorescence intensity became insignificant (saturated). Then, first-order rate constants K for oxidation product generation were derived from the linear regression of $-\ln(1 - (F_{fin} - F_{obs})/(F_{fin} - F_{ini}))$ (F_{fin}, fluorescence intensity at the saturated point; F_{obs}, observed; F_{ini}, initial) versus the reaction time. For CO analytes with a concentration below 1% in air, the reactions were conducted inside a large airtight container which supported excess CO, and reaction mixtures were transferred into a fluorescence cuvette to record the fluorescence spectra after a certain time.

Animal Experiments. Male rats (Orient Yeungnam branch, SD strain) aged 3−4 weeks (~200 g) were used in the CO exposure experiments in this work. For all entries, whole blood was sampled from the abdominal vein via syringe after the animal's death, and heparin sodium was added to prevent coagulation (~1 mg/10 mL blood). For the blank (control) sample, a rat was euthanized at 1 atm of CO_2 (within 3 min), and its blood was sampled and kept at 0 °C. For the 100% HbCO (assumed) sample, a rat was euthanized with 1.0% CO in air (within 11 min), and its blood was sampled and kept at 0 °C. For experimental samples, rats were exposed to 0.10% CO in air (gas box, flow rate 2−5 L/min), and after a certain exposure time, a rat was removed from the CO atmosphere and euthanized at 1 atm of CO_2 (within 3 min) before blood sampling. The whole blood was immediately subjected to HbCO analysis with MPVC-I and MPVC-II.

A simple diffusion method was used to determine the HbCO level in the blood. First, a small glass vial was placed in a large glass vial, with each containing a magnetic stirring bar. Then, 2 mL of probe solution under preset standard conditions was injected into the small vial, and 1 mL of 10 wt % H_2SO_4 (aq) was injected into the large glass vial. Then, 1 mL of whole blood was injected into the large glass vial carefully, and its cap was tightly closed before being subjected to magnetic stirring at room temperature. The volume of air inside the closed vial was approximately 16 mL. After 1 h, the cap was opened, and the fluorescence spectra of the solution in the small vial were recorded.

◼ RESULTS AND DISCUSSION

Initially, we sought to determine the optimum conditions for azidocarbonylation reaction after the design and synthesis of MPVC-I by tuning the proportions of the solvent, catalyst, ligand, and additive (Table S3−1). Herein, we utilized the apparent kinetic constant k to evaluate the response of this fluorescent probe system toward CO by forcing a first-order reaction pattern in terms of absolute fluorescence intensity because of the possible parallel (carboxylation) or serial (Curtius rearrangement) side reactions. Whenever the fluorescence enhancement stopped, we assumed that the reaction had ended. Upon the addition of 10 μM of MPVC-I to 1 equiv (equiv) of Pd(OAc)2 and 20 μM of Xantphos in PBS (phosphate buffer saline, 10 mM), the mixture showed weak 557 nm emission upon 424 nm excitation, but in the presence of 200 equiv of azide (azidation did not occur due to the inactive nature of MPVC−Pd-N3 reductive elimination[32]), the fluorescence decreased to almost zero. After bubbling 1 atm of CO into the azide-containing system, fluorescence emission was rapidly enhanced by 80−100-fold at 552 nm. Surprisingly, in the absence of any organic solvent, ligand, or additive, azidocarbonylation still proceeded, at a significant rate of 0.0683 min^{-1} (entry 2). Nevertheless, with 0.5% of DMSO, the reaction rate increased by approximately 50% (entry 4), probably because DMSO solvated more ligands and/or provided additional coordination with Pd(II);[37] more DMSO did not make a big difference (entries 8 and 9 compared with 5) but did turn on a small amount of initial fluorescence. For the ligands, it seemed that only triaryl phosphines were active (entries 5 and 14−20); specifically, PPh3 and P(o-MeC6H4)3

gave fast reaction rates, but the final fluorescence intensities were approximately half those of the other entries, possibly due to the Staudinger reaction.[38] Additionally, all bidentate phosphine ligands resulted in good conversion, in contrast to the 0−6% yield obtained by Grushin and co-workers.[32] Pd(dba)2 did not give significant turn-on fluorescence, presumably due to Pd^0 aggregation (entry 28). Overall, using various trials, we precisely tuned the concentrations of catalyst, ligand, and additives and fixed our standard CO sensing conditions, as in entry 5 (Figure 3).
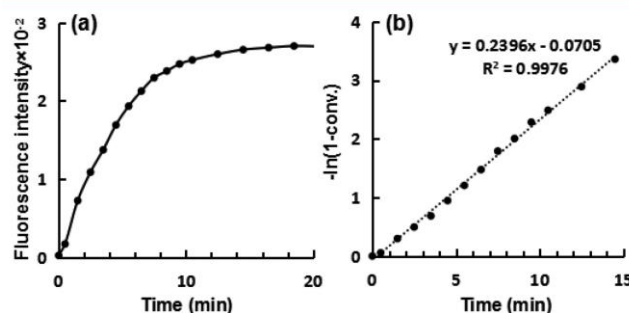

Figure 3. Fluorescence−time curve (a) and first-order kinetics curve (b) of MPVC-I under standard conditions. Conv. (apparent conversion) refers to $(F_{fin} - F_{obs})/(F_{fin} - F_{ini})$ (F_{fin}, fluorescence intensity at final saturated point; F_{obs}, observed fluorescence intensity; F_{ini}, fluorescence intensity at initial point).

Using the aforementioned standard conditions, we screened the selectivity for susceptible species of two probes. Except for the slight enhancement of fluorescence by cysteine and cyanide, species other than CO gave no obvious response to MPVC-I. However, in the presence of various species, sulfides, cysteine, cyanide, thiocyanate, and ferrocyanide inhibited the enhance-ment of fluorescence because they poisoned the palladium catalyst. Iodide also behaved as a strong ligand to Pd(II). This is a common limitation of sensing systems based on transitional metal mediated reactions (Figures 4a,b). Nevertheless, for gaseous samples, H_2S and HCN can be easily masked by Hg^{2+} and acidic MnO_4^-, respectively, thus making CO subject to detection. Compared to MPVC-I, the apparent kinetic response upon the fluorescence intensity change of MPVC-II was much faster.[39] In general, for CO concentrations above 1% in air, the very strong initial fluorescence of MPVC-II turned off so fast that its rate constant could not be measured accurately. In this respect, it was not necessary to optimize either the ligands or additives. Similarly, Hg^{2+}, sulfide, and cyanide containing species poisoned the Pd(II) catalytic center (Figures 4c,d). However, the introduction of sulfide, thiolcyanate, or iodide caused fluorescence quenching of MPVC-II and CO, which we attribute to the subtle interaction between the Pd(II) complex/aggregate and the fluorophore. Moreover, Hg^{2+} would not have a significant effect because of the higher sensitivity of MPVC-II's Truji-Trost reaction. Owing to this special phenomenon, if both MPVC-I and MPVC-II are applied in an analysis, CO in the presence of sulfide can still be detected, which alleviates the limitation of Pd(II)-based chemosensors to some extent. Specifically, MPVC-II itself shifts its yellow fluorescence emission to cyan in the presence of hypochlorite, presumably due to oxidative electron transfer.

CO-mediated reaction processes (Figure 1b) were elucidated by liquid chromatography (LC) and mass spectrometry (MS; Figure 5). For the azidocarbonylation of MPVC-I, after the
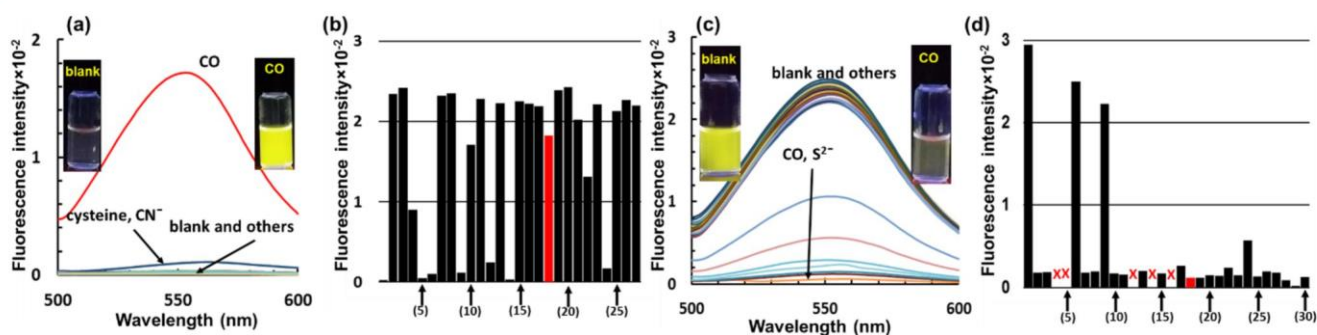
Figure 4. Emission spectra (λ_{ex} = 424 nm) of MPVC-I (10 µM; a) and MPVC-II (2 µM; c) in the presence of 200 µM of chemical species (indicated) under standard conditions. Fluorescence intensity (λ_{ex} = 424 nm and λ_{em} = 550 nm) of MPVC-I (10 µM; b) and MPVC-II (2 µM; d) in the presence of 200 µM of chemical species (indicated) and 1 atm/1% of CO (air balance) under standard conditions. All data were taken after 30 min of incubation. Species used: (2−5) NH4F, NaBr, NaI, Na2S; (6−10) L-cysteine, Na2SO3, formalin, NaCN, NaNO2; (11−15) HCOONa, KSCN, NaH2PO2, K3[Fe(CN)6], H2O2; (16−20) NaClO, NaNO3, 1 atm/1% CO, Cd(NO3)2, CoCl2; (21−25) Cr(NO3)3, CuSO4, FeCl3, HgCl2, MnCl2; (26−30) NiCl2, Zn(OAc)2, NaN3, Na2CO3, NaOAc.
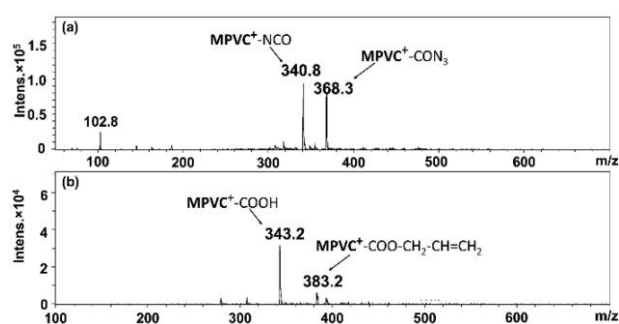

Figure 5. (a) LC-MS graph of MPVC-I in the presence of 1 atm of CO at a retention time of 7.5 min. (b) MS graph of MPVC-II in the presence of 1% CO. Analysis was performed after 10 min from analyte introduction.

introduction of 1 atm of CO for 10 min under standard conditions, the signals of [MPVC$^+$-NCO] and [MPVC$^+$-CON3] appeared at the retention time of 7.5 min, even though the Curtius rearrangement may occur either in solution or on the column. For MPVC-II, exposure to 1% CO for 10 min led to a significant conversion to [MPVC$^+$-COOH].[40] Moreover, the final product of isocyanate hydrolysis and subsequent decarboxylation, [MPVC-NH2], was synthesized separately and gave no fluorescence in the pH 7.4 buffer. The above results confirmed that azidocarbonylation of MPVC-I and deallylation of MPVC-II were responsible for the enhancement and quenching of the fluorescence, respectively.

Then, a lower pressure of CO was investigated using standard conditions. Under 10% (1.0×10^5 ppm) of CO, azidocarboylation of MPVC-I appeared to follow first-order kinetics, with a rate constant k = 0.155 min^{-1} (R^2 = 0.990), indicating that carbonyl insertion was not the rate-determining step. Under 1.0% of CO, the reaction became saturated after 250 min, giving a much lower k of 0.0236 min^{-1} (R^2 = 0.995) in the first 16 min and a k of approximately 0.0100 min^{-1} (R^2 = 0.995) thereafter. When the CO level dropped from 0.50% to 0.10%, the reaction rate decreased drastically (Figure 6a) as the CO insertion began to dominate the reaction process, replacing the oxidative addition. As the temperature increased under 0.10% of CO, within 10 min from the reaction initiation, the fluorescence enhancement exhibited a huge difference, but there was not a significant difference after 60 min at 55 and 90 °C (Figure 6b). By shifting the temperature to 90 °C, MPVC-I could sense CO concentrations as low as 50 ppm with 5-fold fluorescence enhancement after 30 min (Figure 6c), which is the maximum permissible exposure in the workplace for a duration of 8 h, as established by OSHA (Occupational Safety and Health Administration).[41] The Truji-Trost type deal-lylation of MPVC-II proceeded much faster under similar conditions (Figure 6d). Under 0.10% CO, the fluorescence intensity decreased as much as 60% within 5 min, but under 0.010% and 50 ppm of CO, an obvious fluorescence quenching was observed after 30 min of incubation. Since Pd(0) behaves as a catalyst for the Truji-Trost reaction and the Pd(II) reduction to Pd(0) is most likely the rate-determining step under low levels of CO, we did not expect the MPVC-II sensing system to exhibit regular kinetic behavior. The limits of

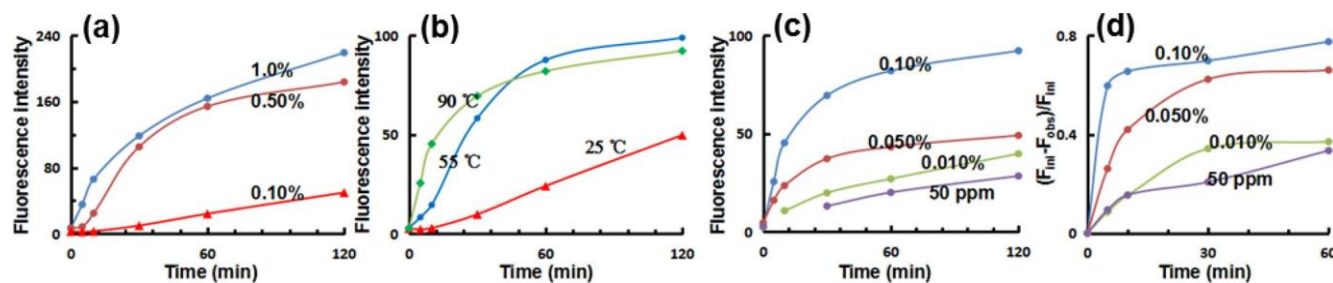Figure 6. Fluorescence intensity versus time for (a) MPVC-I + different concentrations of CO at 25 °C, (b) MPVC-I + different temperatures at 0.10% (1.0×10^3 ppm) of CO, and (c) MPVC-I + different concentrations of CO at 90 °C. (d) (F_{ini} − F_{obs})/F_{ini} of MPVC-II + different concentrations of CO at 25 °C. F_{ini}, initial fluorescence intensity; F_{obs}, observed fluorescence intensity.

detections of MPVC-I (90 °C) and MPVC-II (25 °C) were calculated to be 4.3 and 6.9 ppm, respectively, at 30 min incubation time.[42]

After an on-site investigation of the CO sensing behaviors of MPVC-I and MPVC-II, we used these two probes to determine the carboxyhemoglobin (HbCO) level in the animals' blood. Upon liberating CO from the whole blood of rats in a sealed container by acid, the exposing probe solution exhibited a certain fluorescence response. In the case of MPVC-I, the HbCO level was saturated after 30 min of 0.100% (1.00×10^3 ppm) CO exposure (Figure 7), which was almost
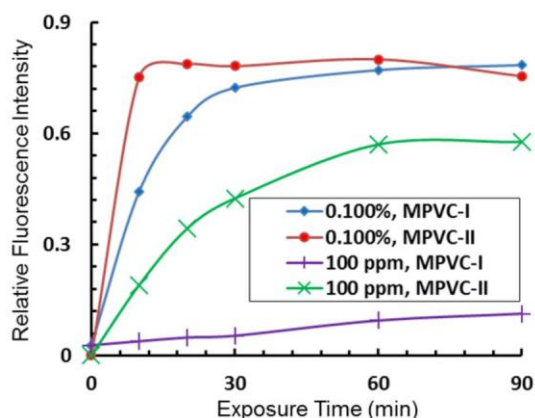

Figure 7. Relative fluorescence intensity of MPVC-I and MPVC-II toward rat exposure time to 0.10% CO in air. Probe solutions for analyses were prepared under preset standard conditions, and the blood was immediately subjected to analysis after sampling. The incubation time was 60 min. $(F_{ini} - F_{obs})/F_{ini}$ of MPVC-I + different levels of CO at 25 °C. The relative fluorescence intensity is F_{obs}/F_{fin} for MPVC-I and $(F_{ini} - F_{obs})/F_{ini}$ for MPVC-II (F_{ini}, initial fluorescence intensity; F_{obs}, observed fluorescence intensity; F_{fin}, fluorescence intensity of 100% HbCO entry). Each spot was taken from the average of two parallel experiments.

consistent with the results of Benignus and Annau.[43] However, for MPVC-II, because of this system's ultrasensitivity, this CO level could not exhibit much difference with varying exposure times. When the CO level for exposure dropped to 100 ppm, MPVC-II became more sensitive for the corresponding HbCO measurement and showed saturation after 60 min of exposure. Comparatively, MPVC-I did not exhibit much fluorescence enhancement due to the low CO concentration. Moreover, the behavior of whole blood did not seem to change up to 8 h storage at 0 °C (Figure 8). On the basis of the CO exposure experiments, we can claim with confidence that MPVC-I and MPVC-II are suitable for the qualitative or semiquantitative determination of the relatively higher and lower HbCO levels from a CO-poisoned individual's blood using either the naked eye or a spectrofluorometer. To improve this sensing system, the empty space of the diffusion container could be reduced, which would use a smaller amount of the blood sample and a shorter incubation time and would offer a more convenient and rapid way of CO poisoning diagnosis and forensic investigation.

Last but not the least, all MPVC-containing compounds were confirmed to be two-photon excitable, regardless of their fluorescent intensity behavior (SI sections 5 and 6), under irradiation of an 800 nm femtosecond laser. Moreover, the cytotoxicity upon mouse fibroblast cells of both probes were measured to be low, especially MPVC-II (SI section 7).
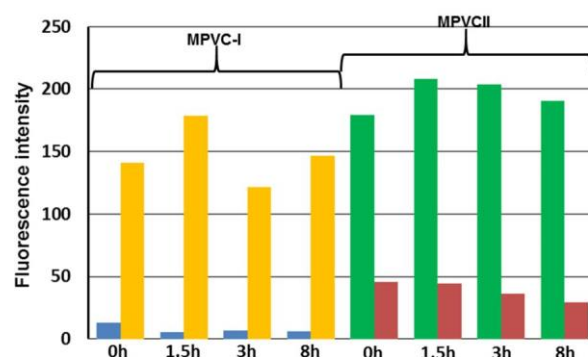

Figure 8. Fluorescence intensities of MPVC-I and MPVC-II subjected to blood samples that were stored at 0 °C for varying times (indicated). Left bar (blue and green) for blank; right bar (yellow and brown) for 100% HbCO.

However, due to the "turn-off" of MPVC-II and the complex reaction mechanism of MPVC-I, neither of these was suitable for bioimaging. Nevertheless, the synthetically feasible MPVC must be able to serve as favorable building blocks for two-photon excitable probes to be designed in the future.

CONCLUSIONS

In this work, we designed and synthesized two-photon excitable fluorescent probes MPVC-I and MPVC-II for sensing gaseous CO. By linking these two probes together, it became possible to overcome the interference of general poisons of a transition metal catalytic center to some extent, which made the probe appropriate for analyzing complicated mixed gases. Addition-ally, the azidocarbonylation reaction was extensively studied in the optimization of the CO sensing condition. Moreover, these two probes were demonstrated to be applicable for determining the HbCO level in an animal's whole blood. We believe that this work will be helpful for the further advancement of fluorescent CO sensors, cost-effective two-photon excitable sensors, palladium-catalyzed carbonylation reaction method-ology, and CO related blood tests.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssen-sors.8b00083.

Additional characterizations of all related compounds, detailed information on spectroscopic studies, and detailed animal experimental procedure (PDF)

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Notes
The authors declare no competing financial interest.

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(39) Five volume percent of acetonitrile in PBS was used as the solvent in order to enhance the contrast of fluorescence intensity before and after analyte introduction.

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