ChemPubSoc Europe

DOI: 10.1002/chem.201502191

CHEMISTRY A European Journal Communication

Fluorescence |*Hot Paper*|

A Boron Dipyrromethene (BODIPY)-Based Cu^{II}–Bipyridine Complex for Highly Selective NO Detection

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Abstract: A BODIPY-containing Cu^{II}–bipyridine complex for the simple selective fluorogenic detection of NO in air and in live cells is reported. The detection mechanism is based on NO-promoted Cu^{II} to Cu^{I} reduction, followed by demetallation of the complex, which results in the clearly enhanced emission of the boron dipyrromethene (BODIPY) unit.

In recent years, environmental awareness has grown due to social dissatisfaction with the state of the environment.[1] As a result, more restrictive environmental laws have been introduced. In this context, air pollution is one of the major problems in urban areas, whose main sources of pollutants are the combustion processes of fossil fuels used in power plants, vehicles, and other incineration processes. The main combustion-generated air contaminants are nitrogen oxides (NO_x), considered primary pollutants of the atmosphere as they are responsible for photochemical smog, acid rain, and ozone layer depletion.[2] On the other hand, NO is a well-known bioactive molecule that participates in a wide range of bioregulatory and immune response processes.[3]

For these reasons, intensive experimental research is being conducted for NO monitoring, and several analytical techniques, such as electrophoresis, electron paramagnetic resonance (EPR) or GC-mass spectroscopies, chemiluminescence, or electrochemical methods, have been developed to detect this hazard.[4] Even though these methods offer certain benefits, they also show some limitations which typically involve poor specificity and the use of expensive experimental apparatus, which restrict their application in practice. Recently, the development of fluorogenic probes has gained growing interest as an alternative to classical instrumental procedures.[5] In this context, fluorogenic probes are especially appealing because they allow simple detection in situ or/and at site, usually without any sample pre-treatment. Moreover, changes in emission can be detected by simple equipment and it is a very sensitive detection technique. Changes in emission properties can also be detected by the naked eye, which makes this procedure very appealing.

Several probes for the fluorogenic detection of NO have been reported. For instance, poorly fluorescent vicinal diamines can be transformed into triazoles by NO, which results in a significant increase in fluorescence.[6] Other sensing protocols based on NO-induced ring closure,[7] deamination reactions,[8] or NO-induced aromatization,[4b] have been recently studied. Metal-based probes that take advantage of the reactivity of NO at the metal site have also been reported; for instance, nitric oxide sensing has been accomplished with Co^{II}, Fe^{II}, Ru^{II}, Rh^{II}, and Cu^{II} complexes.[9] However, some of these probes display drawbacks, such as dependence on pH or the tendency of certain dyes to form aggregates. Given the significance of NO to human health and diseases, most probes have been tested to monitor NO production in vivo, whereas very few studies have been conducted into nitric oxide detection in air.

Following our interest in designing probes for the fluorogenic detection of gases,[10] we focused on the potential use of BODIPY for designing NO sensing probes. BODIPY is a well-known fluorophore that possesses valuable optical characteristics, such as absorption and fluorescence transitions in the visible spectral region with high molar absorption coefficients and fluorescence quantum yields, good stability, and no dependence on pH.[11] Yet despite these advantageous BODIPY fluorophore optical properties, as far as we know, no metal complexes based on BODIPY derivatives have been reported for fluorogenic NO detection.

The design of our probe was based on two concepts. First, it is known that the BODIPY derivatives that bear a 4-pyridine moiety attached to the *meso* position induce fluorescence quenching when the pyridine group is protonated, through the formation of a weakly emissive charge-transfer state.[12] We

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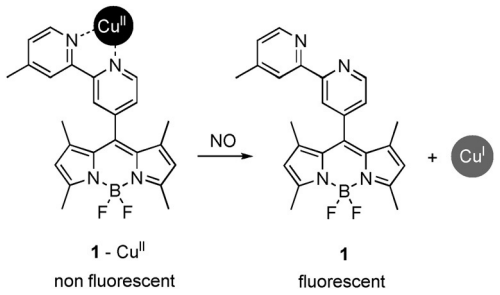
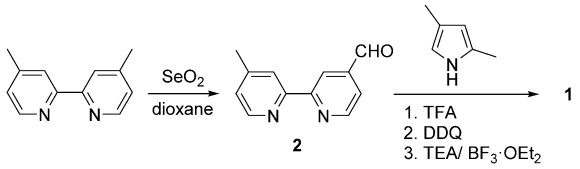
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201502191.

postulated that similar BODIPY-bipyridine derivatives that coordinate metal cations would also be poorly emissive. On the other hand, it has been well-established that the copper centre in Cu^{II} complexes undergoes reduction to Cu^{I} in the presence of NO, followed by demetallation. Based on these concepts, we prepared complex **1**-Cu^{II} (see Scheme 1). This complex is expected to be poorly fluorescent, whereas significant fluorescence enhancement is expected to be selectively observed in the presence of NO.



Scheme 1. Complex **1**-Cu^{II} and schematic outline of the sensing paradigm.

The synthesis of BODIPY derivative **1** began with the preparation of bipyridine aldehyde **2** by the oxidation of 4,4′-dimethyl-2,2′-bipyridine with selenium oxide.[13] Compound **1** was readily obtained by the condensation of **2** with 2,4-dimethylpyrrole following standard BODIPY synthesis procedures (see Scheme 2).[14] BODIPY **1** was characterised by ^{1}H and ^{13}C NMR spectroscopy and MS (see the Supporting Information for details).



Scheme 2. Synthesis of ligand **1**. DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; TEA = triethylamine.

Solutions of **1** (1.0×10^{-4} M in acetonitrile) showed an intense absorption band at $\lambda = 500.5$ nm (log $\varepsilon = 7.6$) and an intense emission band at $\lambda = 520.5$ nm ($\lambda_{exc} = 478$ nm, $\Phi = 0.570$). Moreover, fluorescence quenching was observed when Cu^{II} (as nitrate salt) was added (see Table 1). This was in agreement with the coordination of the Cu^{II} cation to the bipyridine binding group. This metal complexation likely triggered an intramolecular charge-transfer process in the excited state from the BODIPY core to the bipy–Cu^{II} unit, and the resulting charge-transfer state became weakly fluorescent.[12]

The formation of 1:1 complexes between **1** and Cu^{II} was confirmed through titration experiments in acetonitrile. The stability constant for the formation of the corresponding **1**-Cu^{II} complex was calculated from fluorescence titration experiments (Figure 1) with the SPECFIT program.[15] A value of log $K = 4.9 \pm 1.6$ was determined. Additional experiments demonstrated that addition of Cu^{I} to the acetonitrile solutions of **1** induced no changes in either the UV or emission spectra, which suggests this cation's poor coordination with the bipy unit. It was also found that the emission quenching of **1**, which was similar to that found for **1** with Cu^{II}, was observed in the presence of diamagnetic cation Zn^{II}. This finding indicates that the poor emission observed for the **1**-Cu^{II} complex was not due to the presence of a paramagnetic cation, but resulted from the coordination of the metal with the pyridine moiety attached to the *meso* position of the BODIPY fluorophore. Finally, the fluorescence emission intensity of **1** was evaluated at different pH values, with only minor changes in the pH range between 4–12 (see the Supporting Information).

Table 1. The UV/Vis and fluorescence data of **1** and **1**-Cu^{II}.

	λ_{abs} [nm]	log ε	λ_{em} [nm]	Φ[a]
1	500.5	7.6	520.5	0.570
1-Cu^{II}	504.0	7.1	519.0	0.071

[a] Quantum yields were calculated using rhodamine B in ethanol as a standard ($\Phi_{EtOH} = 0.49$, $\lambda_{exc} = 478$ nm).

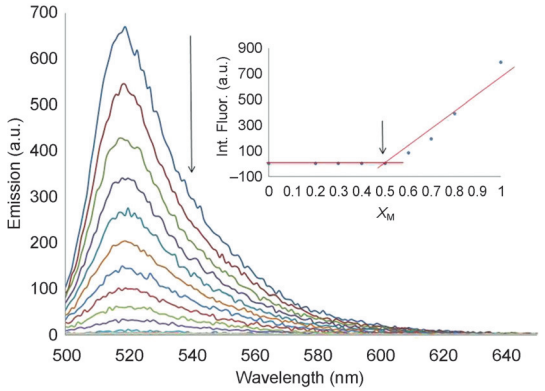


Figure 1. Fluorescence spectra of **1** (acetonitrile, 1.0×10^{-5} M) with increasing amounts of Cu^{II}. Inset: molar ratio graphic.

Complex **1**-Cu^{II} was easily isolated by simply stirring **1** with one equivalent of $Cu(NO_3)_2$ in EtOH/water for 2 h. The resulting yellow precipitate was recrystallised from EtOH/water, filtered, and dried. In order to evaluate the sensing ability of **1**-Cu^{II}, the water/acetonitrile (9:1 *v/v*) mixtures of the complex (1.0×10^{-5} M) were exposed for 5 min to N_2 atmospheres, which contained increasing quantities of NO, and the corresponding emission spectra were recorded. The presence of NO greatly increased the fluorescence emission at 520.5 nm ($\lambda_{exc} =$ 478 nm), which was attributed to the presence of free **1** (Figure 2). In fact, exactly the same emission band was observed for the solutions of **1** in the water/acetonitrile (9:1 *v/v*) mixtures. The fluorogenic sensing ability of probe **1**-Cu^{II} was also observed by the naked eye. In particular, a bright-green emission was clearly seen when the solutions of **1**-Cu^{II} exposed to NO were illuminated at 254 nm with a conventional UV lamp (Figure 2).

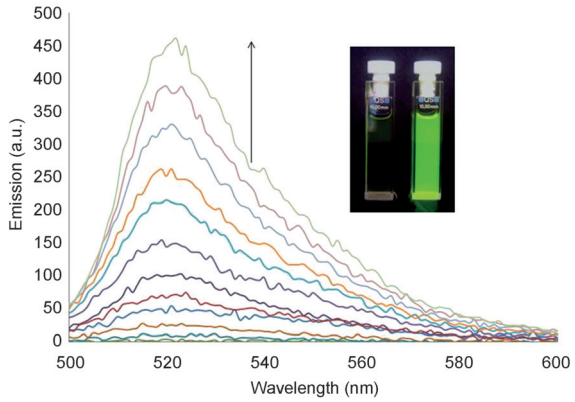


Figure 2. Fluorescence spectra of complex **1**-Cu^{II} (water/acetonitrile 9:1 *v/v*, 1.0×10^{-5} M) in the presence of increasing NO concentrations from 0 to 80 ppm after 5 min. Inset: the visual changes ($\lambda_{ex}=254$ nm) observed for **1**-Cu^{II} before and after exposure to 1 ppm of NO.

From the titration studies, limits of detection (LOD) of 3 ppm (from fluorescence) and 0.5 ppm (from UV/Vis) were calculated by the 3Sb1/S procedure (Sb1 is the standard deviation of the blank solution and S is the slope of the calibration curve).[16]

In addition, **1**-Cu^{II} was recovered after the oxidation of Cu^{I} to Cu^{II} induced by atmospheric oxygen. In particular, the regeneration of the **1**-Cu^{II} probe was achieved after keeping the **1**-Cu^{II}-NO mixture in NO-free air. This process was repeated at least five times with only minimal loss of fluorescence intensity (Figure 3).

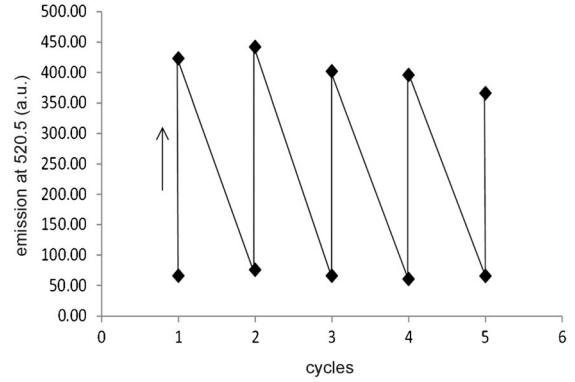


Figure 3. The cycles of emission intensity of **1**-Cu^{II} (1.0×10^{-5} M in acetonitrile) at $\lambda_{em}=520.5$ nm ($\lambda_{exc}=500.5$ nm) upon successive exposures to NO (5 min) and to a NO-free air atmosphere (24 h).

Encouraged by the sensing ability of BODIPY derivative **1**-Cu^{II}, we decided to move another step forward and we studied the potential use of the probe for detecting NO in air. To this end, a membrane that contained **1**-Cu^{II} was designed. In a typical preparation, polyethylene oxide (*Mw* 400000 Dalton) was slowly added to a solution of **1**-Cu^{II} in dichloromethane. The mixture was stirred until a highly viscous mixture was formed and finally poured into a glass plate. The system was kept in a dry atmosphere for 24 h to obtain the corresponding sensing membrane.

In a typical assay, the membrane was placed into a container that held NO (1 ppm). After 5 min, fluorescence clearly enhanced, as observed by the naked eye with a conventional 254 nm UV lamp (Figure 4). A LOD of 0.3 ppm to the naked eye after 10 min for sensing NO in air was determined.[17]

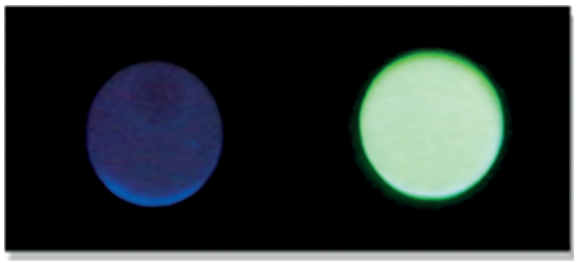


Figure 4. Emission of a polyethylene oxide membrane of **1**-Cu^{II} ($\lambda_{ex}=254$ nm) (left) and after exposure to 1 ppm of NO in air for 5 min (right).

One important issue for designing probes for pollutant gases is the role played by the potential interferents or false-positive outcomes produced by other species. When bearing this in mind, the potential reactivity of **1**-Cu^{II}-containing membranes to other hazardous gases (i.e., NO_2, CO_2, H_2S, SO_2) at a concentration of up 100 ppm in air was also studied. The probe was also tested in the presence of vapours of acetone, hexane, chloroform, acetonitrile, and toluene, and up to a concentration of 100 ppm in air. No emission changes were observed in the presence of any of these chemical species. Besides, competitive experiments demonstrated that the membrane was able to detect NO in a mixture that also contained the aforementioned gases and vapours (see the Supporting Information for details). Such behaviour indicated that **1**-Cu^{II} was a suitable highly selective probe for detecting nitric oxide in complex air samples.

Finally, we also assessed the ability of the probe to detect NO release in cells (Figure 5). In order to further demonstrate the biological application of BODIPY–copper complex **1**-Cu^{II}, RAW 264.7 macrophages were stimulated for 18 h with *Escherichia coli* lipopolysaccharide to induce NO synthase (iNOS).[18] After washing cells with phosphate buffered saline, **1**-Cu^{II} (10 μM) was added and NO release was initiated by the addition of L-arginine as a substrate of iNOS. RAW 264.7 macrophages stimulated with only arginine or only **1**-Cu^{II} showed low fluorescence, whereas the addition of arginine in the presence of **1**-Cu^{II} significantly increased fluorescence, which demonstrates this compound's ability to detect NO released by cells. Even though these results indicate that other components of the complex cellular matrix did not interfere with the detection results, further sensing experiments were carried out with some possible biological competitors in Cu^{II} binding, such as cysteine, glutathione, or histidine. No significant increment in fluorescence was observed in any case (see the Supporting Information, Figure S14)

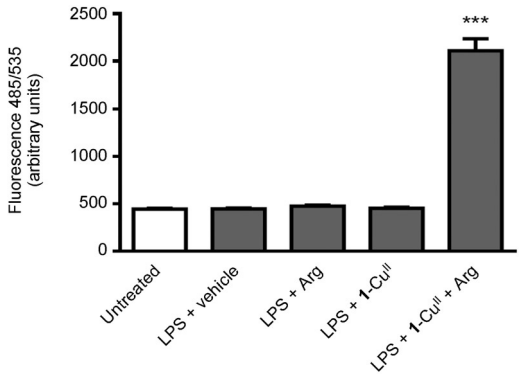


Figure 5. NO detection in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS). After cell washing, **1**-Cu^{II} (1.0×10^{-5} M), dissolved in water/acetonitrile 95:5 *v/v* (vehicle), was incubated for 30 min with cells, and Arginine (Arg) 100 μM was added to induce NO release. Data are expressed as mean ± SEM ($n=8$–12). ***$p<0.001$ compared to the LPS + vehicle-treated cells. Dunnett's t test for multiple comparisons.

Figure 6 also shows the fluorescence images of the RAW 264.7 cells stimulated with LPS and the incubation of **1**-Cu^{II} (10 μM) in the absence or presence of arginine (100 μM). Emission was clearly enhanced in the cells that contained NO. In a parallel experiment, absence of the cytotoxicity of **1**-Cu^{II} under the same conditions was demonstrated by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (see the Supporting Information).

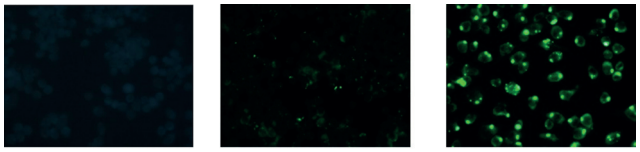


Figure 6. Fluorescence imaging of RAW 264.7 stimulated with LPS and incubated for 30 min with **1**-Cu^{II} (10 μM) in the presence or absence of Arginine (Arg) 100 μM.

In summary, we report herein the synthesis and sensing properties of a new BODIPY–Cu^{II} complex for NO detection. Probe **1**-Cu^{II} contains a BODIPY fluorophore and a bipyridine unit capable of coordinating Cu^{II}. Reduction of Cu^{II} to Cu^{I} mediated by NO resulted in demetallation and a significant enhancement of the emission of the BODIPY unit. NO sensing was achieved in solution and in the gas phase. In particular, probe **1**-Cu^{II} in the polyethylene oxide membranes was satisfactorily used to monitor NO levels in air. Furthermore, the response of probe **1**-Cu^{II} to NO was selective in air and other hazardous gases, such as NO_2, CO_2, H_2S, SO_2, and vapours of different solvents at concentrations of up 100 ppm were unable to induce emission modulations. A LOD as low as 0.3 ppm to the naked eye after 10 min for NO sensing in air was calculated. Probe **1**-Cu^{II} was also suitable for NO detection in live RAW 264.7 macrophages with no cytotoxic effects.

Acknowledgements

The authors thank the DGICYT and European FEDER funds (MAT2012-38429-C04-01 and MAT2012-38429-C04-02) and the Generalitat Valenciana (PROMETEOII/2014/047) for support. SCSIE (Universidad de Valencia) is gratefully acknowledged for all the equipment employed.

Keywords: BODIPY · cells · fluorescence · NO detection · quimiodosimeter

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Received: June 4, 2015
Published online on August 3, 2015