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A BODIPY-based bipyridine-Cu^{II} complex for the highly selective detection of NO

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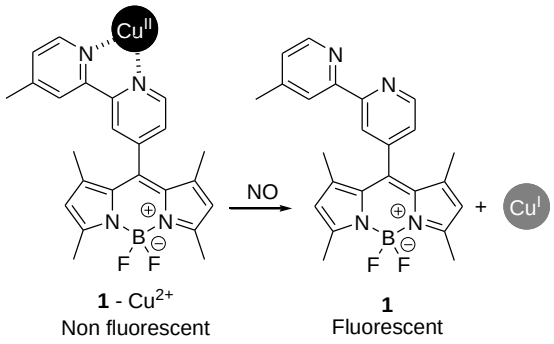
A new BODIPY-containing Cu^{II}-complex for the simple fluorogenic detection of NO in air and in live cells is reported.

In recent years environmental awareness has grown due to the social dissatisfaction with the state of the environment.[1] As a result, more restrictive environmental laws have been introduced. In this context, air pollution constitutes one of the major problems in urban areas being the main sources of pollutants 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) which are considered the primary pollutants of the atmosphere, since they are responsible for the photochemical smog, the acid rain and ozone layer depletion.[2] On the other hand, NO is a well-known bioactive molecule which participates in a large variety of bioregulatory and immune response processes.[3]

For these reasons intensive experimental research is being carried out for NO monitorization and several analytical techniques such as electrophoresis, electron paramagnetic resonance (EPR) or GC-mass spectroscopies, chemiluminescence or electrochemical methods have been developed for the detection of this hazard.[4] Even though these methods have certain benefits they also show some limitations typical involving poor specificity and the use of expensive experimental apparatus which restrict their application in practice. Recently, the development of fluorogenic probes has gained increasing 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 using simple equipment and it is a very sensitive detection technique. In addition changes in emission properties can be detected to the naked eye making this procedure highly attractive.

Several probes for the fluorogenic detection of NO have been reported. For instance poorly fluorescent vicinal diamines can be transformed to triazoles by NO resulting in a strong increase of fluorescence.[6] This mechanism is the basis of most fluorogenic NO probes. Moreover some new sensing protocols based NO-induced ring closure,[7] de-amination reactions,[8] or NO-induced aromatization[9] have been recently studied. Besides metal-based probes that take advantage of the reactivity of NO at the metal site have also been reported, and for instance nitric oxide sensing has been accomplished using Co^{II}, Fe^{II}, Ru^{II}, Rh^{II} and Cu^{II} complexes.[10] However some of these probes display some drawbacks such as dependence on the pH or tendency of certain dyes to form aggregates. Moreover because of the significance of NO to human health and diseases most of the probes have been tested to monitor NO production *in vivo*, whereas very few studies have been devoted to nitric oxide detection in air.



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

Following our interest in the design of probes for the fluorogenic detection of gases,[11] we have focused our attention to the potential use of BODIPY for the design of NO sensing probes. BODIPY is a well-known fluorophore which possesses valuable optical

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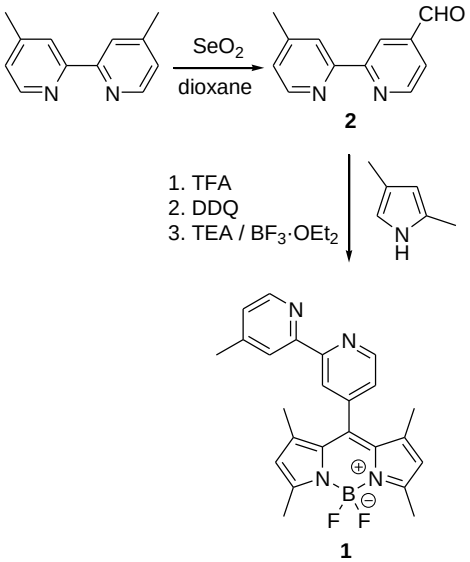
characteristics, such as absorption and fluorescence transitions in the visible spectral region with high molar absorption coefficients and fluorescence quantum yields, good stability and no dependence on pH.[12] However, despite these advantageous optical properties of the BODIPY fluorophore there are not, as far as we know, metal-complexes based in BODIPY derivatives for the fluorogenic detection of NO.

The design of our probe was based in two concepts. First it is know that BODIPY derivatives bearing a 4-pyridine moiety attached to the meso position induced quenching of the fluorescence when the pyridine group protonated through the formation of a weakly emissive charge-transfer state.[13] We postulated that similar BODIPY-bipyridine derivatives coordinating metal cations would also be poorly emissive. On the other hand, it is well established that the copper centre in Cu^{II} complexes undergo reduction to Cu^{I} in the presence of NO followed by demetallation. Based in these concepts we prepared complex **1**-Cu^{II} (see Scheme 1). This complex is expected to be poorly fluorescent whereas a significant fluorescence enhancement it is expected to be selectively observed in the presence of NO.

Synthesis of the BODIPY derivative **1** started with the preparation of the bipyridine aldehyde **2** by oxidation of 4,4'-dimethyl-2,2'-bipyridine with selenium oxide.[14] From **2**, **1** was readily obtained by condensation of **2** with 2,4-dimethylpirrole following standard procedures for BODIPY synthesis (see Scheme 2).[15] BODIPY **1** was characterized by ^{1}H-NMR, ^{13}C-NMR and MS (see Supporting Information for details).



Scheme 2. Synthesis of **1**.

Solutions of **1** (1.0 x 10^{-4} M in acetonitrile) showed an intense absorption band at λ = 500.5 nm (log ε = 7.6) and an intense emission band at λ = 520.5 nm (λ_{exc} = 478 nm, Φ = 0.570). Moreover when Cu^{II} (as nitrate salt) was added, a quenching of the fluorescence was observed (see Table 1). This was in agreement with the coordination of the Cu^{II} cation to the bipyridine binding group. It is very likely that this metal complexation triggered an intramolecular charge-transfer process in the excited state from the BODIPY core to the bipy-Cu^{II} unit, being the resulting charge-transfer state weakly fluorescent.[13]

Table 1. 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 (Φ_{EtOH} = 0.49) as standard (λ_{exc} = 478 nm).

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) using SPECFIT program.[16] A value of log K = 4.9 ± 1.6 was determined. In additional experiments it was observed that addition of Cu^{I} to acetonitrile solutions of **1** induced no changes in the UV or in the emission spectra suggesting a poor coordination of this cation with the bipy unit. Moreover it was also found that a similar emission quenching of **1** that that found for **1** with Cu^{II}, was observed in the presence of the diamagnetic cation Zn^{II}, suggesting that the poor emission observed for the **1**-Cu^{II} complex was not due to the presence of a paramagnetic cation but consequence of the coordination of the metal with the pyridine moiety attached to the meso position of the BODIPY fluorophore.

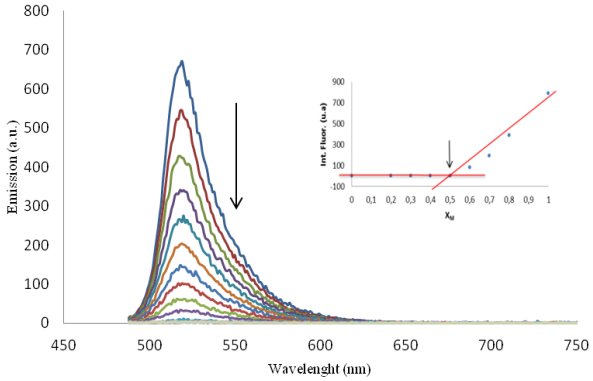


Figure 1. Fluorescence spectra of **1** (acetonitrile, 1.0 x 10^{-5} M) with increasing amounts of Cu^{II}. Inset: mole ratio graphic.

Finally complex **1**-Cu^{II} was easily isolated by simple stirring **1** with 1 equivalent of $Cu(NO_3)_2$ in EtOH-water for 2 h. The resulting yellow precipitate was recrystallized from EtOH-water, filtered and dried (see Supporting Information for details).

In order to evaluate the sensing ability of **1**-Cu^{II}, a N_2 atmosphere containing increasing quantities of NO were bubbled on water:acetonitrile (9:1 v/v) mixtures of the complex (1.0 x 10^{-5} M) for 5 min, and the corresponding emission spectra were recorded. The presence of NO induced a strong increment of the fluorescence emission at 520.5 nm, (λ_{exc} = 478 nm) that was attributed to the presence of free **1** (Figure 2). In fact, exactly the same emission band was observed for solutions of **1** in 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 solutions of **1**-Cu^{II} exposed to of NO were illuminated with at 254 nm using a conventional UV lamp (see Figure 2).

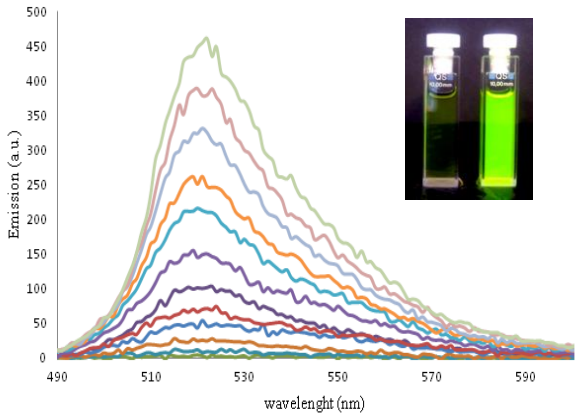


Figure 2. Fluorescence spectra of complex **1**-Cu^{II} (water: acetonitrile 9:1 v/v, 1.0 x 10^{-5} M) in presence of increasing concentration of NO from 0 to 80 ppm after 5 min. Inset: visual changes (λ_{ex} = 254 nm) observed for **1**-Cu^{II} before and after exposure to 1 ppm of NO.

From titration studies a limit of detection (LOD) of 3 ppm (from fluorescence) and 0.5 ppm (from UV-vis) were calculated using the 3Sb1/S procedure (Sb1 is the standard deviation of the blank solution and S is the slope of the calibration curve).[17]

In addition, it was found that **1**-Cu^{II} was recovered after the oxidation of Cu^{I} to Cu^{II} induced by atmospheric oxygen. In particular we observed that regeneration of the **1**-Cu^{II} probe was achieved after keeping the **1**-Cu^{II}-NO mixture under NO-free air. This process can be repeated at least five times with only a small loss of fluorescence intensity (see Figure 3).

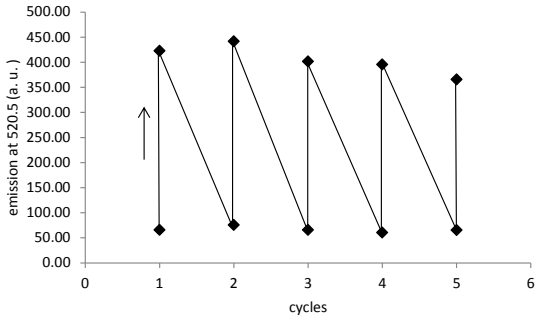


Figure 3. Cycles of emission intensity of **1**-Cu^{II} (1.0 x 10^{-5} M in acetonitrile) at λ_{em}= 520.5 nm (λ_{exc}= 500.5 nm) upon successive exposures to NO (5 min) and to NO-free air atmosphere (24 h).

Encouraged by the sensing ability of the BODIPY derivative **1**-Cu^{II}, we decided to take a further step and studied the potential use of the probe for the detection of NO in air. To this end, a membrane containing **1**-Cu^{II} was designed. In a typical preparation polyethylene oxide (Mw 400,000 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 in order to obtain the corresponding sensing membrane.

In a typical assay the membrane was placed into a container holding NO (1 ppm). After 5 min a clear enhancement of the fluorescence was observed by the naked eye by using a conventional 254 nm UV lamp (Figure 4). From fluorescence measurements it was determined a LOD to the naked eye of 0.3 ppm after 10 min for the sensing of NO in air.

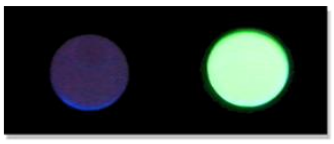


Figure 4. Emission of a polyethylene oxide membrane of **1**-Cu^{II} (λ_{ex} = 254 nm) (left) and after exposure to 1 ppm of NO in air for 5 min (right).

One important issue in relation to the design of probes for pollutant gases is the role played by potential interferents or false-positive outcomes produced by other species. Bearing this in mind, the potential reactivity of **1**-Cu^{II}-containing membrane 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. Moreover the probe was also tested in the presence of vapours of acetone, hexane, chloroform, acetonitrile and toluene also 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 also containing the gases and vapours commented above. Such behaviour indicated that **1**-Cu^{II} was a suitable highly selective probe for the detection of nitric oxide in complex air samples.

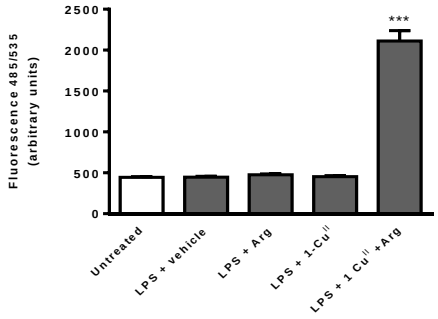
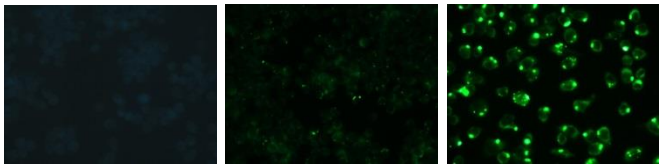


Figure 5. NO detection in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS). After cell washing, **1**-Cu^{II} (1.0 x 10^{-5} M) dissolved in water:acetonitrile 95:5 v/v (vehicle) was incubated for 30 min with cells and Arginine (Arg) 100 µM was added to induce NO release. Data are expressed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

Apart of the detection of NO in air we have also assessed the ability of the probe to detect NO release in cells. In particular to further demonstrate the biological application of the BODIPY-copper complex **1**-Cu^{II}, RAW 264.7 macrophages where stimulated for 18h with *Escherichia coli* lipopolysaccharide in order 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 addition of L-Arginine as substrate of iNOS. Stimulated RAW 264.7 macrophages with only arginine or **1**-Cu^{II} showed low fluorescence whereas the addition of arginine in the presence of **1**-Cu^{II} produced

a significant increase of fluorescence, demonstrating the ability of this compound to detect NO released by cells (see Figure 5).



LPS LPS + **1**-Cu^{II} LPS + **1**-Cu^{II} +

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

Moreover Figure 6 shows fluorescence images of RAW 264.7 cells stimulated with LPS and incubated **1**-Cu^{II} (10 μM) in the absence or presence or arginine 100 μM. A clear enhancement of the emission was found in cells containing NO. In a parallel experiment, the absence of cytotoxicity of **1**-Cu^{II} in 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 Supporting Information).

Conclusions

In summary, we have reported herein the synthesis and sensing properties of a new BODIPY-Cu^{II} complex for the detection of NO. Probe **1**-Cu^{II} contains a BODIPY fluorophore and a bipyridine unit able to coordinate 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 both in solution and in gas phase. In particular probe **1**-Cu^{II} in polyethylene oxide membranes were satisfactorily used for the monitoring of 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 a concentration of up 100 ppm were unable to induce emission modulations. A LOD to the naked eye as low as 0.3 ppm after 10 min. for NO sensing in air was calculated. Moreover probe **1**-Cu^{II} was also suitable for NO detection in live RAW 264.7 macrophages without cytotoxic effects.

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