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A coumarinylaldoxime as a specific sensor for Cu^{2+} and its biological application

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ABSTRACT

In this Letter we present a new probe, (*E*)-7-(diethylamino)-2-oxo-2*H*-chromene-3-carbaldehyde oxime (**JB**), which can detect Cu^{2+} ions in HEPES buffer under physiological conditions. Benesi–Hildebrand and Job plots demonstrate that the stoichiometry of the Cu^{2+} complex formed is 2:1. Possible interference with other analytes was examined, and the decrease of the fluorescence of **JB** at 510 nm when it reacts with Cu^{2+} was shown to be highly selective. This probe accumulates in the plasmalemma of human neuroblastoma SH-SY5Y cells. Molecular dynamics (MD) simulations revealed that **JB** interacts with the lipid bilayer at the level of the glycerol moieties.

Novel colorimetric and fluorescent probes for metal ions have found actual and potential applications in different areas of science.[1] Copper is the third most abundant trace element in many living organisms including humans, and it is involved in redox processes,[2] besides catalyzing the formation of reactive oxygen species (ROS) that are capable of damaging biomolecules leading to the connection of the cellular toxicity of copper ions with serious diseases.[3–6] Cu^{2+} complexes have also attracted considerable interest in nucleic acid chemistry due to their various applications following the discovery of their chemical nuclease activity.[7,8]

In this work, the novel fluorescence probe **JB** was synthetized in three steps as shown in Scheme 1. 4-(Diethylamino)-2-hydroxybenzaldehyde (**1**) was condensed with diethyl malonate in a Knoevenagel reaction, cyclized and decarboxylated in one step to afford 3-amino-7-hydroxycoumarin (**2**), the coumarin was formylated (Vilsmeier-Haack) to obtain compound **3**, which was condensed with hydroxylamine hydrochloride to afford **JB**, characterized by ^{1}H NMR and ^{13}C NMR spectroscopy (Fig. S3A and B; Supporting information).

All absorption and emission spectral studies were performed in 20 mM HEPES buffer, pH 7.4, at room temperature, while the corresponding metal chlorides were used as the source of the metal cations. The absorption spectrum of **JB** shows a maximum at 430 nm (Fig. S4; Supporting information), a molar absorptivity (ε) of 23,058 L mol^{-1} cm^{-1} and an emission band at 510 nm (Fig. S5; Supporting information). The quantum yield of this probe is 0.043, with a lifetime (τ) of 2.78 ns and a Stokes shift of 3648 cm^{-1}.

In order to evaluate the selectivity of **JB**, its fluorescence spectra were recorded with different metal ions added (Hg^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+} or Cd^{2+}). Among these ions, only Cu^{2+} significantly affected the fluorescence intensity of **JB** (Fig. 1). Fig. 2 shows the Cu^{2+} concentration-dependent emission fluorescence spectrum of **JB** (20 μM). When excited at 430 nm, the emission fluorescence intensity at 510 nm decreases about 8-fold upon increasing the concentration of Cu^{2+} from 0 to 100 μM (Fig. 2).

A Benesi–Hildebrand graph of the fluorescence data (Fig. S6; Supporting information) was non-linear, indicating that the stoichiometry of the Cu^{+2} complex formed is different from 1:1. The binding stoichiometry of the **JB**-Cu^{2+} complex was determined from the Job plot (Fig. S7; Supporting information). Maximum emission intensity was observed when the mole fraction of Cu^{2+} was close to 0.34, which indicates the formation of a 2:1 complex between **JB** and Cu^{2+} at a total concentration of 100 μM. This was further confirmed by the appearance of a peak at *m*/*z* 292.9497 assignable to $[2MX+Cu]^{+2}$ in the ESI spectrum (Fig. S8; Supporting

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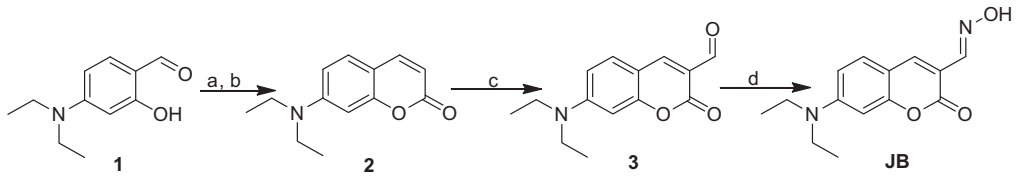


Scheme 1. Synthetic route to **JB**. Reagents and conditions: (a) Diethyl malonate, piperidine, AcOH, EtOH, reflux, 6 h; (b) HCl, AcOH, reflux, 24 h; (c) $POCl_3$, DMF, 60 °C, 24 h; (d) hydroxylamine hydrochloride, Et_3N, rt 1 h.

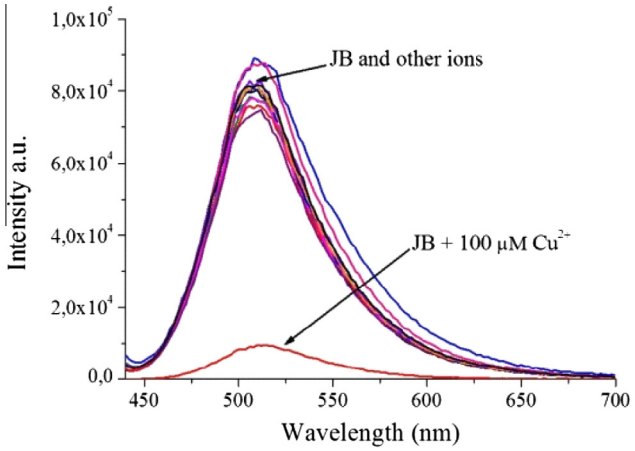


Figure 1. Fluorescence spectra of **JB** (20 μM) alone and in the presence of several different metal salts (Hg^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+} or Cd^{2+}; μM) in 20 mM HEPES buffer, pH 7.4.

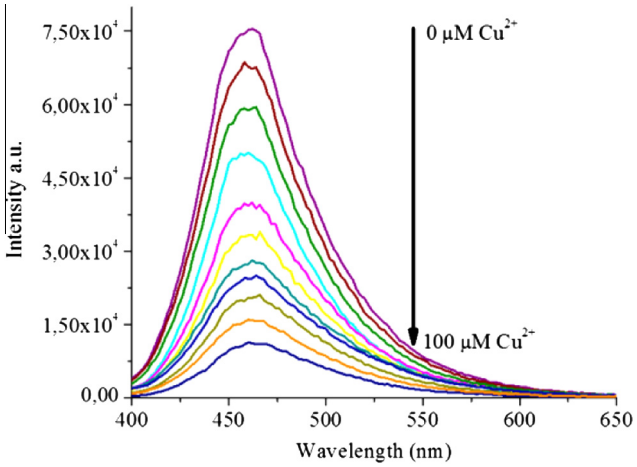
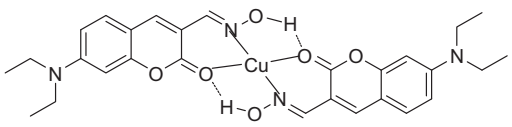


Figure 2. Fluorescence emission spectra (λ_{ex} = 460 nm) of **JB** (10 μM) upon addition of $CuCl_2$ (0–100 equiv) in 20 mM HEPES buffer, pH 7.4.



Scheme 2. Possible binding mode of **JB** with Cu^{2+}.

information). A possible binding mode between **JB** and Cu^{2+} is proposed in Scheme 2.

To further check the practical applicability of **JB** as a Cu^{2+}-selective fluorescent sensor, we carried out competitive experiments in

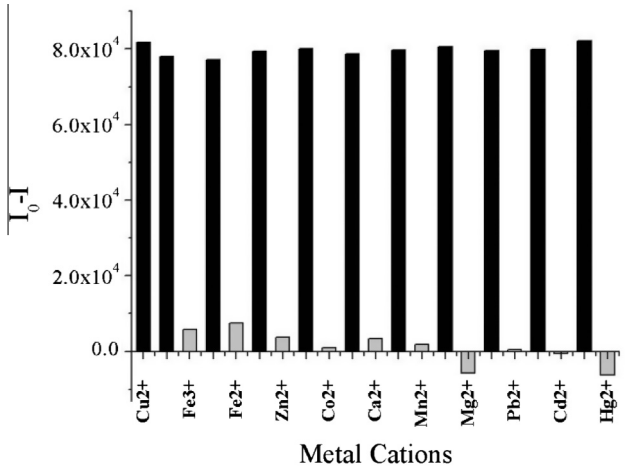


Figure 3. Selectivity of the fluorescence quenching of **JB** by Cu^{2+} in HEPES buffer pH 7.4. Gray bars indicate the fluorometric responses of **JB** with 10 equiv of Fe^{3+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Pb^{2+}, Cd^{2+}, and Hg^{2+}. Black bars represent the florescence response after addition to the same solutions of 10 equiv of Cu^{2+}.

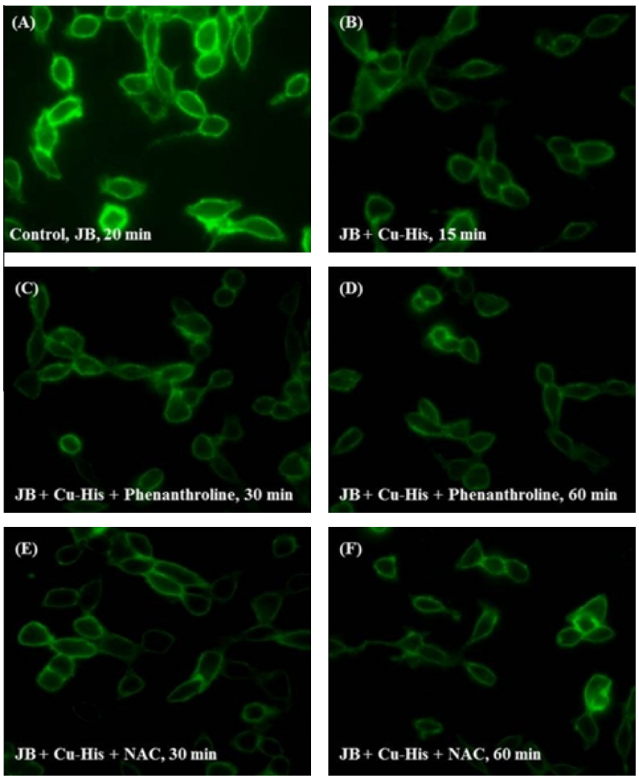


Figure 4. In vitro tests of the potential of **JB** as a cell membrane probe for Cu^{2+}. (A) SH-SY5Y cells were incubated with **JB** (10 μM, 20 min), washed, and the basal fluorescence measured. (B) The cells were then incubated with Cu-His (200 μM, 15 min). (C) And (D) fluorescence after adding 1,10-phenanthroline (20 μM, 30 and 60 min). (E) And (F) fluorescence after adding NAC (15 mM, 30 and 60 min). The fluorescence was recorded by epifluorescence microscopy, 63× objective.

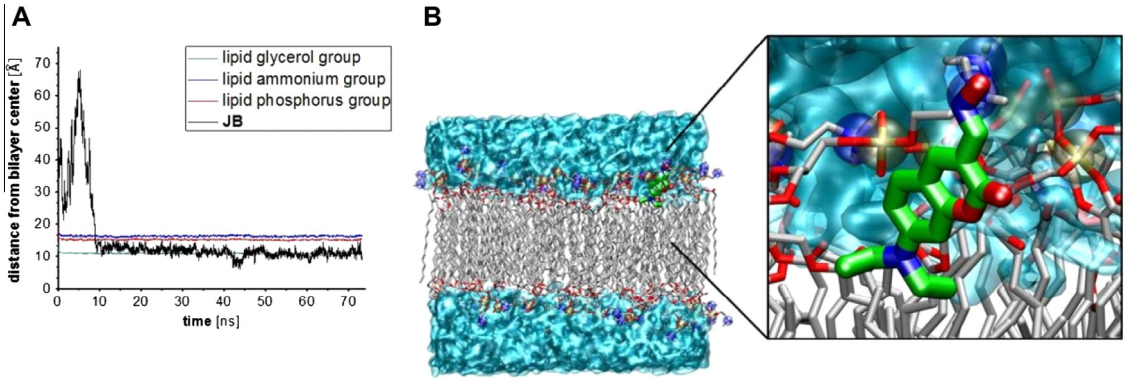


Figure 5. The dependence of the position of the **JB** center of mass as a function of time of unconstrained MD simulation. (A) The location of **JB** is depicted by a black curve. The positions of the ammonium (blue curves), phosphate (red curves), and glycerol (cyan curves) moieties of the lipid bilayer are also plotted. (B) Typical snapshot of a POPC bilayer containing **JB** from MD simulations. A zoomed snapshot is also shown.

the presence of 100 μM Cu^{2+} mixed with other metal ions at 100 μM. As shown in Figure 3, these experiments demonstrate that none of the selected ions interfere to any obvious extent with the detection of Cu^{2+}.

Fluorescent probes available for copper are many.[9] However, bifunctional copper-chelating compounds with preference for a cell structure are scarce, and even more so if they exhibit preference for the cell membrane. Some fluorescent heterocycles posessing a diethylamino group have shown affinity for the cell membrane,[10] and our group has recently published a Letter confirming this property.[11] To determine if the test compounds were retained in the cell membrane, SH-SY5Y cells were incubated additionally with compound FM (results under publication), a fluorescent membrane probe based on 7-[(6-bromohexyl)oxy]-2*H*-chromen-2-one. Observing the change in fluorescence caused by different treatments, **JB** (green fluorescence) was concentrated in the periphery of the cells, as with **FM** (red). The co-localization experiments resulted in the emission of yellow light, showing the simultaneous presence of both compounds in the plasma membrane (Fig. S9; Supporting information).

JB was therefore evaluated for its potential as a probe for Cu^{+2} in SH-SY5Y human neuroblastoma cells. The cells were initially incubated with **JB** under physiological conditions and then subsequently treated with the histidine–Cu^{+2} complex as a source of Cu^{2+}. Fluorescence was monitored using epifluorescence microscopy (Fig. 4). The fluorescence was significantly quenched (Fig. 4B) following addition of histidine-Cu^{+2}, with most of the fluorescent species residing in the cell membrane. The reversibility of the probe was examined by adding 1,10-phenanthroline (a molecule with high affinity for the metal) (Fig. 4C and D) and *N*-acetylcysteine (NAC), to observe the possible detection of thiols (Fig. 4E and F),[11] but no reversal was observed. In addition, this probe is fairly soluble in an aqueous medium and has a high affinity for copper, which could increase its importance for implementing a detection method.

A molecular dynamics (MD) simulation was performed in order to study the incorporation of **JB** into the lipid phase of the cell membrane.[11] The software used and the conditions of the MD simulations are described in the Supplementary material. **JB** was initially placed in the bulk water, far from the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane. After 8 ns of simulation, **JB** was incorporated into the lipid membrane (Fig. 5A). This process corresponds to the passive diffusion of the probe toward the membrane surface. After its incorporation, the more polar region of **JB** (the (hydroxyimino)methyl group) was oriented away from the surface of the lipid bilayer (Fig. 5). Analysis of the positions occupied by **JB** during the MD simulation shows that this molecule does not enter into the depths of the membrane, and interacts only with the groups near the surface (Fig. 5B and zoom). We also evaluated the effect of **JB** on the orientation of the membrane lipids. To do this we calculated the order parameter S_{CD} of the acyl chains of sn-1 and sn-2 POPC. Comparing pure POPC with the phospholipid adsorbing **JB**, we found that the order of the sn-1 chains in the system containing **JB** was increased (Fig. S10; Supplementary data), but sn-2 showed increased order only at the carbon atoms near the surface.

Conclusions

We have developed and characterized a new oxime-based probe that is highly sensitive and selective for Cu^{2+} ion detection in an aqueous environment at pH 7.4. This compound accumulates selectively in the plasmalemma of human neuroblastoma SH-SY5Y cells due to its interaction via the diethylamino group, as indicated by molecular dynamics (MD) simulations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.12.033.

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