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A near-infrared fluorescent sensor for detection of cyanide in aqueous solution and its application for bioimaging†

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A new NIR fluorescent sensor based on an amine-substituted heptamethine cyanine dye displayed a highly selective fluorescence enhancement with cyanide in aqueous solutions, and was applied for the imaging of anthropogenic and biogenic cyanide.

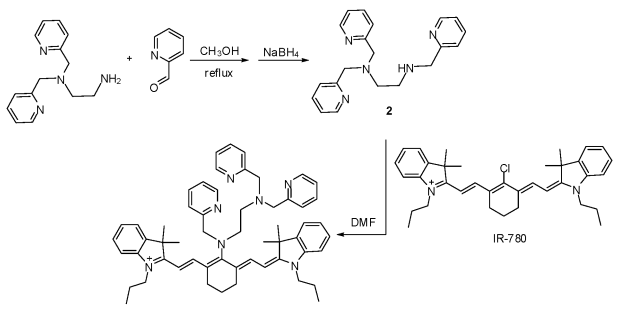
HCN produced by *Pseudomonas aeruginosa* (PA) is considered to be involved in the pathogenesis of CF lung disease.[1] The imaging of cyanide *in vivo* systems can be a valuable tool for elucidating the mechanism of the bacterial cyanogenesis as well as assessing the detriment of HCN to CF patients. However, monitoring the amount of cyanide that is produced by the microorganism in the lungs is not an easy task because sputum samples are not easily obtained from either pediatric patients or small animal models such as mice. Therefore, it is highly needed to develop a fluorogenic method,[2] which is simple and convenient to detect cyanide *in vivo*. As part of this effort, optical probes that can detect cyanide with ease and high sensitivity have recently been exploited.[3] Surprisingly, most of these probes were designed based on UV-visible fluorophores or sensed cyanide only in organic solvents, which limit their applications on fluorescence imaging for clinical diagnosis.

Near-infrared (NIR) dyes have received considerable attention for fluorescent bioimaging due to their advantages over UV and visible fluorophores.[4] In 2005, Peng *et al.* reported that amine-substituted tricarbocyanines that have absorption maximum at a much shorter wavelength thus lead to much larger Stokes shifts than those of oxygen/sulfur-substituted cyanines.[5] Since then, amine-substituted heptamethine cyanine dyes have been developed as fluorescent sensors for metal ions and pH value.[6] Even now, no NIR fluorescent probe for imaging cyanide has been reported.

Among the various systems designed to detect anions, sensors utilizing the affinity of anions for copper have attracted special attention.[7] We report here a novel NIR fluorescent sensor containing an amine-substituted heptamethine cyanine dye and a copper complex moiety, which selectively sense cyanide in aqueous solution. Furthermore, studies of biological applications using *Caenorhabditis elegans* demonstrated that this system can be employed for the imaging of cyanide.

For the synthesis of a sensor precursor, as shown in Scheme 1, *N*,*N*,*N*′-tris(pyridin-2-ylmethyl)ethane-1,2-diamine (**2**) was obtained firstly according to the similar methods reported previously.[8] **2** was then reacted with IR-780 in DMF to give **1** in 15% yield. Compound **1** comprises a N_5-donor coordination sphere bearing three 2-pyridylmethyl moieties, which lead to strong affinity between compound **1** and Cu^{2+}. This design also assures strong fluorescence quenching in the presence of copper ion due to the short distance between the fluorophore and metal binding site. When the Cu^{2+} concentration increases, the peak intensity of the absorption maximum at 718 nm of this sensor decreases with the concurrent formation of a new peak at 743 nm (Fig. 1, top). When 1 equiv. of copper ion was added to **1** in aqueous solution, complete fluorescence quenching was also observed (Fig. 1, bottom). The binding constant of **1** with Cu^{2+} was determined to be 1.1×10^6 M^{-1} based on the fluorescence titration experiments. Other metal ions, such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and Ag^+, induced negligible fluorescence quenching effects, which implies that **1** has potential as a Cu^{2+}-selective NIR sensor (Fig. S1, see ESI†).

As complete quenching happened when 1 equiv. of copper ion was added to **1**, the sensor **1–Cu^{2+}** was prepared *in situ* by mixing compound **1** with $Cu(ClO_4)_2$ at a 1 : 1 ratio in aqueous solution. The extinction coefficients of compound **1** and **1–Cu^{2+}** were calculated to be 1.12×10^5 M^{-1} cm^{-1} and 1.16×10^5 M^{-1} cm^{-1}. Using the IR 125 as standard, the fluorescence quantum yields



Scheme 1 Synthesis of compound **1**.

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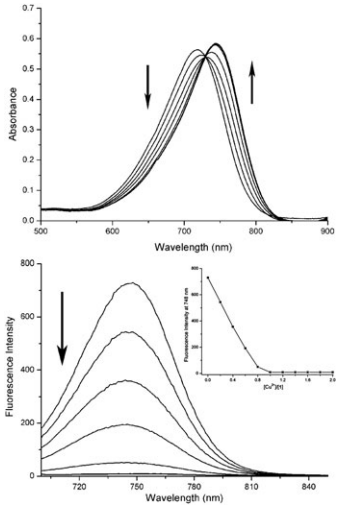

Fig. 1 (Top) Absorbance spectra of **1** (5 μM) with various concentrations of Cu^{2+} (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μM) in 20 mM HEPES buffer (pH 7.4, 0.5% CH_3CN). (Bottom) Fluorescence spectra of **1** (5 μM) with addition of various concentrations of $Cu(ClO_4)_2$ (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μM) in 20 mM HEPES buffer (pH 7.4, 0.5% CH_3CN) with an excitation at 680 nm. Inset: the plot of emission intensity at 748 nm *vs.* $[Cu^{2+}]/[\mathbf{1}]$.

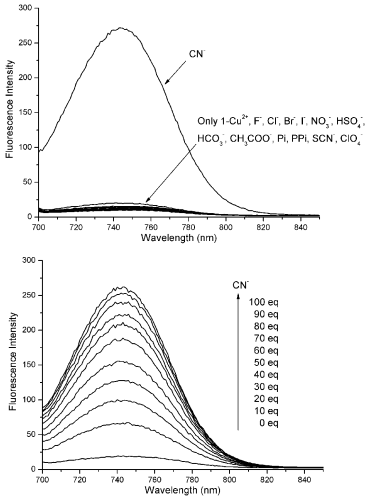

Fig. 2 (Top) Fluorescence changes of **1–Cu^{2+}** (5 μM) with various anions (100 equiv.) in 20 mM HEPES buffer (pH 7.4) (λ_{ex} = 680 nm). (Bottom) Fluorescence spectra of **1–Cu^{2+}** (5 μM) with different concentrations of CN^- (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 equiv.) in 20 mM HEPES buffer (pH 7.4, 0.5% CH_3CN).

of compound **1** and **1–Cu^{2+}** were calculated to be 0.026 and 0.005, respectively. When CN^- was added gradually to the solution containing **1–Cu^{2+}**, the peak intensity of the emission at 748 nm of this sensor increased, and the emission change upon the addition of 100 equiv. cyanide was approximately 14-fold (Fig. 2, bottom). Adding 1000 equiv. CN^- to the solution containing **1–Cu^{2+}** (0.5 μM), the fluorescence can be recovered completely (Fig. S2, see ESI†). Using 0.5 μM **1–Cu^{2+}**, the detection limit of the probe was evaluated to be 5 μM with a signal-to-noise ratio of 3 (Fig. S3, see ESI†). In the presence of 100 mM Cl^- or HCO_3^-, the fluorescence of **1–Cu^{2+}** still increases remarkably in the presence of 100 equiv. CN^- (Fig. S4, see ESI†). In addition, the fluorescence intensity at 748 nm has no remarkable change in the pH 2.7–10.4 range (Fig. S5, see ESI†), which means that the disturbances of proton and high concentration of physiologically relevant anions are neglectable in the present system.

The selective response of the probe to cyanide was then investigated. Various anions (100 equiv.), such as F^-, Cl^-, Br^-, I^-, NO_3^-, HSO_4^-, HCO_3^-, CH_3COO^-, Pi (phosphate), PPi (pyrophosphate), SCN^- and ClO_4^-, did not respond to the probe (Fig. 2, top). In contrast, cyanide induced the revival of fluorescence of the probe, resulting in "*Turn-On*" type sensing of cyanide. As cyanide reacts with copper ions to form stable $[Cu(CN)_x]^{n-}$ species, the quenched fluorescence of system is then recovered upon the addition of cyanide.

The nematode *C. elegans* was used to assess its feasibility for applications as a NIR sensor to imaging. It is an important experimental model in various fields including microbiology[9] and environmental toxicology.[10] As shown in Fig. 3, NIR fluorescence was not observed in *C. elegans* without the pre-exposure to NaCN (Fig. 3a). When the nematodes were exposed to 1 μM NaCN, NIR fluorescence was only observed in the pharynx and intestine (Fig. 3b). However, when the concentration of NaCN was increased to 100 μM, fluorescence was observed throughout the body of the nematodes (Fig. 3c). This suggests that cyanide taken through the mouth can diffuse easily across the intestine, whereas it is difficult for it to diffuse across the cuticle layer on the epidermis that protects the body from harsh environments. Interestingly, NIR fluorescence was observed in the cytosol but not in the nucleus, suggesting that once the toxin reaches the cell it remains in the cytosol where the mitochondria, the target organelle of cyanide, are present (Fig. 3d).

PA is the most important pathogen causing chronic infection in CF patients. It is able to synthesize HCN, a potent inhibitor of cellular respiration. The present study investigated whether cyanide is present in *C. elegans* infected with PA. For the confirmation of bacterial infection in the intestine of the nematodes, *C. elegans* fed on PA14 strain were labeled with GFP (green fluorescent protein) before the imaging. As shown in Fig. 4a, after *C. elegans* were fed with *E. coli* OP50 and then exposed to the sensor, neither green nor NIR fluorescence was observed in the nematodes. In contrast, both green and NIR fluorescence were observed in the nematodes fed on PA14 and exposed to the sensor (Fig. 4b–d). Similarly with the exogenous NaCN, the biogenic cyanide diffused across the intestine and was thus observed throughout the body except

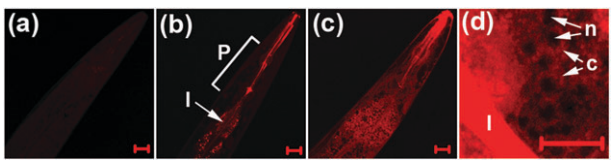

Fig. 3 NIR imaging of NaCN in *C. elegans*. The nematodes on NGM plates were exposed for 4 h to various concentrations of NaCN; (a) no cyanide, (b) 1 μM, (c) 100 μM. (d) The enlarged image of the vicinity of the intestine in the nematode exposed to 100 μM NaCN. All the exposed nematodes were then washed three times with NGM buffer and incubated with the NIR chemosensor (final conc. 100 μM) for 20 min. The scale bars represent 20 μm (P = pharynx; I = intestine; n = nucleus; c = cytosol).

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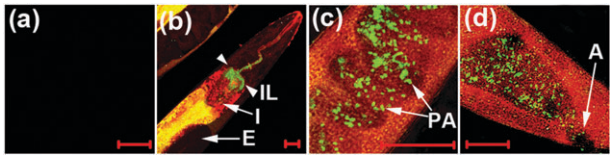

Fig. 4 NIR imaging of cyanide in *C. elegans* infected with a *P. aeruginosa* strain (PA14) labeled with green fluorescent protein (GFP). Before the imaging, the nematodes were fed on either non-infectious *E. coli* OP50 (a) or GFP-labeled PA14 for 2 days (b–d); (b) the anterior end, (c) the medial part, (d) the posterior end of *C. elegans*. The scale bars represent 20 μm (IL = intestinal lumen; I = intestine; E = eggs; PA = PA14-GFP; A = anus).

for the eggs (Fig. 4b). This result indicates that the sensor clearly responded to HCN produced by PA14 in the nematodes. The nematodes fed on PA14 died several days earlier than those fed on *E. coli* OP50, indicating that PA14 infection is responsible for the early death of the PA14-fed nematodes. When the nematodes fed on PA14 were treated with ceftazidime, a β-lactam antibiotic, both the green and NIR fluorescence intensities were significantly reduced, compared to those intensities observed in PA14-infected nematodes without the antibiotic treatment (Fig. 5). This result indicates that bacterial load in the intestine and cyanide production were decreased by ceftazidime. Notably, when ascorbate was added to the sensing system, the fluorescence enhancement was observed (Fig. S6, see ESI†). However, from the imaging experiments, biological reductants did not disturb the determination of anthropogenic and biogenic cyanide.

In conclusion, we have shown that a new water-soluble and NIR fluorescent sensor **1–Cu^{2+}** effectively and selectively recognizes cyanide in aqueous solution. This sensor was also used to visualize the cyanide produced by *P. aeruginosa* in *C. elegans*. These results suggested that the present system has potential as a powerful tool for the imaging of anthropogenic and biogenic cyanide.

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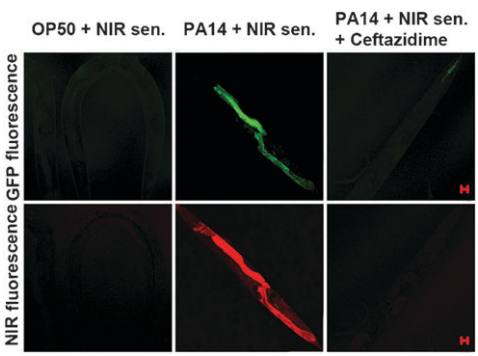

Fig. 5 Visualization of antibiotic efficacy against *P. aeruginosa* infection in *C. elegans* with the NIR sensor. The nematodes were fed on GFP-labeled *P. aerugionosa* strain (PA14) for 2 days. They were then incubated with ceftazidime (200 μg mL^{-1}) for 2 h before the *in vivo* imaging. The scale bars represent 20 μm.

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