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Communication

Cyanide selective *off-on* fluorescent chemosensor with *in-vivo* imaging in 100% water: solid probe preferred over *in-situ* generation†

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A nontoxic fluorescent chemosensor $[Cu(BP)HMB]_2(ClO_4)_2$ (1) synthesized in solid phase, exhibits unprecedented selectivity and sensitivity over the allied *in-situ* complexes to perform fluorescence in "turn-*off-on*" mode for sensing cyanide in 100% aqueous medium under physiological conditions and for *in-vivo* imaging using the nematode *C. elegans*. Below μM detection limit, instantaneous and excellent ratiometric response are also beneficial to detect trace amounts of anthropogenic or biogenic cyanide.

The design and development of chemosensors capable of detecting selective toxic and lethal anionic species are of current research interest in chemistry, biology, medicine and in relation to environmental issues.[1] Among various biologically hazardous anions, cyanide (CN^-) is considered to be the most potent one.[2] According to the World Health Organization (WHO), the maximum acceptable level of CN^- in drinking water is 1.9 μM.[3] However, the widespread use of CN^- in industries and their waste effluents impose serious threat to aquatic environment.[4] Hence, intensive effort should be given for the development of 100% water soluble selective and sensitive CN^- chemosensor with rapid response for effective *in-vivo* detection. The detection principles of such chemosensors are based on mainly H-bonded/self-assembled receptor approach,[1b-c,5] displacement approach[1a,5a,6] or chemidosimeter approach,[5a,7] although majority of them are operative in organic solvents or mixed aqueous solvents and sometimes not even applicable for liquid phase at all.[1,5-7] Considering the advantage-disadvantage parameters of those approaches, it is presumed that the transition metal based cationic fluorescent chemosensors by displacement approach would be the optimal choice to maintain aforesaid all criteria for

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†Electronic Supplementary Information (ESI) available: experimental details and charactarizations. CCDC 960526 (**1**). For ESI and crystallographic data in CIF See DOI: 10.1039/b000000x/

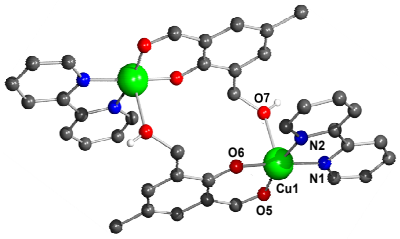
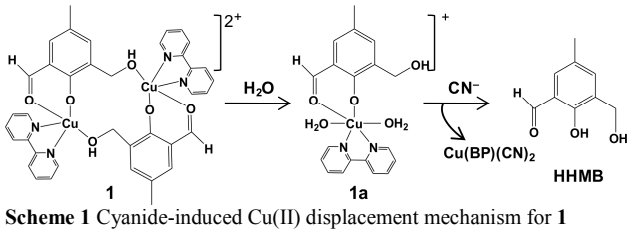


Fig. 1 Molecular view of the sensor **1** with atom numbering scheme. All H-atoms except that for O7 and ClO_4^- are omitted for clarity. Color index: C (black), N (blue), O (red), Cu (green).

CN^- detection in aqueous medium.[1a,5a,6] However, discovery of completely water soluble sensing probe with sensitivity to detect below μM CN^- is still a limiting phenomenon.[5b,6a]

In Cu(II) mediated displacement approach, a fluorescent probe is designed based on a giant organic dye whose fluorogenic activity is totally quenched by the complexation with Cu(II). On exposure to CN^-, decomplexation occurs to form stable $[Cu(CN)_x]^{n-}$ species and fluorogenic activity reappears from the liberated dye.[1a,5a] Unfortunately, owing to scarce solubility of those dyes and their copper complexes in aqueous medium, possibilities of *in-vivo* application are relatively obscure. Generally, toxic organic solvents in different extents are used to solubilize the sensors in aqueous medium, except one rare report is there which operates in 100% aqueous medium with *in-vivo* application.[6a] Again, in most of the cases, sensors were synthesized under *in-situ* condition without solid state isolation.[5-7] Hence the detail analytical comparisons in terms of efficiency, in between the solid probe and that generated *in-situ*, are still missing in the literatures.

Herein, we report the design and synthesis along with detailed structural analysis of a solid fluorescent chemosensor $[Cu(BP)\mathbf{HMB}]_2(ClO_4)_2$ (**1**) (BP = 2, 2'-bipyridine), based on a



Scheme 1 Cyanide-induced Cu(II) displacement mechanism for **1**

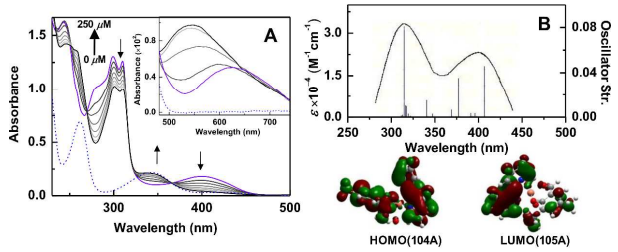


Fig. 2 (A) UV-Vis absorption spectra of **1** (50 μM) in presence of increasing CN^- concentration (0–250 μM) in 20 mM HEPES, pH 7.4. (*Inset*) Spectra of **1** (100 μM) in presence of CN^- (0–500 μM) are shown. The dashed blue line for **HHMB** (50 μM) is used as reference. CN^- free spectrum of **1** is indicated by purple color. (B) TDDFT spectrum (250–450 nm) in water and FMO (below) for 404-nm CT transition of **1a**.

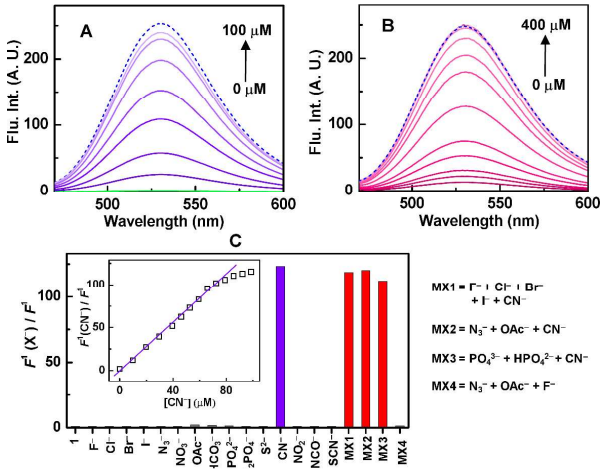


Fig. 3 Fluorescence response of (A) **1** (20 μM), (B) **HHMB** (20 μM) + $Cu(bpy)(ClO_4)_2$ (120 μM) towards increasing CN^- concentration in 10 mM HEPES, pH 7.4. The green spectrum (A) is for **1** in absence of CN^-. The dashed blue line for **HHMB** (20 μM) is used as reference. (C) Ratio of fluorescence intensity of **1** (20 μM) in presence ($F^1(CN^-)$) and absence (F^1) of various anions (100 μM) or mixture of anions (100 μM for each anion) are depicted by bar-diagram. (*Inset*) The ratio for **1** at different CN^- concentrations are plotted. (Excitation: 440 nm)

tiny organic chromophore, 2-hydroxy-3-(hydroxymethyl)-5-methylbenzaldehyde (**HHMB**), operating in "turn-*off*-*on*" mode with high selectivity and sensitivity for recognizing the CN^- in 100% aqueous medium as well as in living biological system. It is noteworthy that in aqueous medium, the dimeric solid probe (**1**) undergoes irreversible change to its monomeric form $[Cu(BP)(\mathbf{HMB})(H_2O)_2](ClO_4)$ (**1a**) which is actually responsible for cyanide attack (*vide infra*) as depicted in **Scheme 1**. The water soluble organic compound **HHMB** was prepared according to the similar method reported previously with some additional modifications[8] (ESI†). It behaves as an organic fluorophore with a moderately high quantum yield (ϕ_F) *ca.* 0.05 in aqueous medium (ESI†). The extent of fluorescence quenching of **HHMB** was determined for *in-situ* complexation with $Cu(ClO_4)_2$, where inadequate quenching in presence of saturating 6-equivalent Cu(II) ions precludes its selection to act as the sensor in "turn-*off*" mode. Interestingly, under similar condition, the fluorogenic activity of **HHMB** was quenched quite efficiently by strategically attaching the strong field ligand BP in Cu(II) precursor as $Cu(BP)(ClO_4)_2$. The strong field character of the BP may enhance the electron transfer process more facile for sufficient quenching of **HHMB** fluorescence to perform appropriate "turn-*off*" mode of sensing (Fig. S1, ESI†). Meanwhile, in absorption titrations, the depletion of 340 nm absorption band of **HHMB** with concomitant formation of a new absorption band *ca.* 400 nm in both cases with the isobestic point *ca.* 367 nm were due to ligand to Cu(II) charge transfer (CT) during complexation (Fig. S2, ESI†). We are fortunate enough to synthesize the more preferred sensor **1** in solid state and characterized the structure by the single crystal X-ray diffraction, where the sensor **1** crystallizes in the space group $P\bar{1}$ with Z=2 to exist as dimer in solid phase (Fig. 1) (ESI†). In aqueous medium, the dimeric core probably breaks up into two monomeric units. The most probable monomeric structure (**1a**) in aqueous solution was optimized in gas phase by DFT calculation using Gaussian 03 program[9] (Fig.S3, ESI†). The pattern of absorption spectrum of **1a** computed from TDDFT calculations[10] in aqueous solution, specially the newly generated band *ca.* 404 nm for HOMO (104A) → LUMO (105A) excitation nicely matches with the experimental spectrum (Figure 2B). The existence of monomer (**1a**) was further supported by the mass analysis of **1** (ESI-MS^+: *m/z* 521.37 for $[\mathbf{1a}H]^+$; 543.36 for $[\mathbf{1a}Na]^+$) (Figure S4A, ESI†). Again, identical CT band *ca.* 400 nm for **1** and corresponding *in-situ* complex, 1:1 stoichiometry in solution confirming from fluorescence quenching measurement and Job's method (Fig. S1,S5, ESI†) indicate that **1** produces same species in aqueous solution which was generated during $Cu(BP)(ClO_4)_2$ *vs.* **HHMB** titration (*vide supra*); although unlike **1**, small residual fluorescence during *in-situ* generation at saturated condition was probably due to some unreacted **HHMB** (Fig. S1A, ESI†). Complete fluorescence quenching of **1** finally renders its applicability to act as the sensor preferable over its *in-situ* generation.

To determine the selective cyanide sensing ability, sensor **1** (20 μM, with respect to **HMB** unit) was used as completely non-fluorescent "turn-*off*" mode in 100% aqueous buffer at physiological pH 7.4 (Fig. 3A). Instantaneous CN^--induced fluorescence intensity was increased sharply *ca.* 10-fold at 10 μM CN^- (Inset, Fig. 3C). The increase of intensity continued until saturation was obtained *ca.* 100 μM of CN^- (Fig. 3A) and we wonder that it took *ca.* 400 μM CN^- to reach the saturation for *in-situ* generated complex of **1** (Fig. 3B). For selectivity, similar experiments were performed under identical conditions with various potentially interfering anions (N_3^-, OAc^-, HPO_4^{2-} *etc.*) as well as bio-disturbing molecules (ATP, glutathione, cysteine, urea *etc.*), and failed to recover any noticeable fluorescence, however, **1** recognized selectively CN^- from the mixture of various other anions/molecules with almost identical accuracy and hence CN^- sensing ability of **1** is confirmed unambiguously. (Fig. 3C, Fig. S6, ESI†). Interestingly, identical fluorescence characteristic of free **HHBM** and that for **1**+CN^- in "turn-*on*" mode, confirms the liberation of free fluorophoric **HHMB** by the CN^--induced decomplexation. Indeed, the identical excited state lifetimes (*ca.* 4.8 ns) for free **HHBM** and sensor **1** containing 5-equivalent CN^- also support this proposition (Fig. S7, ESI†). The maximum increase of emission intensity ratio *ca.* 115-fold by CN^- (Figure 3C), was much higher than those for *in-situ* complexes, *viz. ca.*18 and 4 for $Cu(BP)(ClO_4)_2$ and $Cu(ClO_4)_2$ respectively (Fig. S8, ESI†).

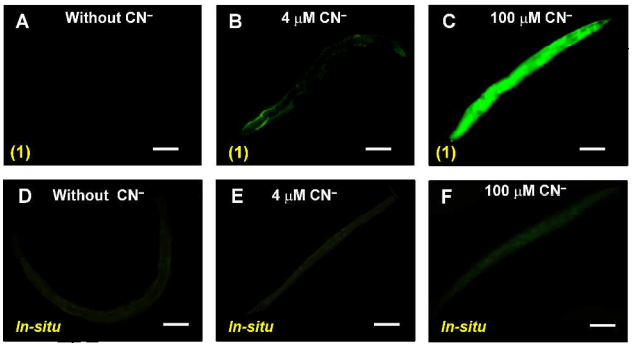


Fig. 4 Fluorescence images of the nematode *C. elegans* exposed to complex **1** (A–C) and *in-situ* complex between **HHMB** and $Cu(BP)(ClO_4)_2$ (D–F) in presence of different CN^- concentration. The phase contrast is identical for all images. The scale bars: 40 μm.

Unlike allied *in-situ* complexes, excellent co-linearity for the change in emission intensity for **1** with CN^- concentration up to 4-equivalent in 100% aqueous medium at physiological pH can be utilized as a good ratiometric chemosensor in biochemical systems (Inset Fig. 3C, Figure S8D, ESI†). The ratiometric value was almost remaining constant in the pH range 6.5–8.0 (Fig. S9, ESI†) as well as insensitive towards other common interfering metal ions (Na^+, K^+, Ca^{2+}, Zn^{2+}, Mg^{2+} *etc.*) present in biological systems. The obtained limit of detection (LOD) *ca.* 0.7 μM is well below the WHO permissible limit (ESI†) and hence **1** can be an excellent choice for even below μM CN^- detection.

The chemical changes during CN^--induced decomplexation were also investigated by the UV-Vis absorption studies. A distinct reduction of the CT band *ca.* 400 nm for **1** was observed with increasing CN^- concentration along with the concomitant formation of a new absorption band at a similar position *ca.* 340 nm of **HHMB** absorption (Fig. 2A). The CN^--induced decomplexation for **1** also accompanied by isobestic points at *ca.* 367, 316, 269 and 251 nm indicates the generation of free **HHMB**. To ascertain the Cu(II) coordination of **1** during displacement, we compared the changeover for CN^--induced d-d absorption profile of **1** and $Cu(BP)(ClO_4)_2$ respectively. In both cases, close resemblance of the d-d absorption spectra confirmed the identical Cu(II) coordination either in the displaced Cu(II) from **1** (Inset Fig. 2A) or in $Cu(BP)(ClO_4)_2$ (Fig. S10, ESI†). Moreover, the absence of characteristic absorption spectrum of free BP for $Cu(BP)(ClO_4)_2$ in presence of CN^- (Fig. S10, ESI†), assured the direct attachment of CN^- to the Cu(II) centre in **1** to produce $[Cu(BP)(CN)_x]^{n-}$. In fact, the ESI mass analysis of **1** in presence of 5-equivelent CN^- confirmed the generation of $Cu(BP)(CN)_2$ (ESI-MS^+: *m/z* 278.68 for $[Cu(BP)(CN)_2Li]^+$) (Fig. S4B, ESI†).

To demonstrate the bio-applicability of sensor **1** for CN^- detection, the nematode *C. elegans* was used for *in-vivo* imaging. It is the most suitable organism for testing the cyanide toxicity of municipal and industrial waste water.[6a] No fluorescence was observed in *C. elegans* when it was exposed to **1** (20 μM) (Figure 4A). When the nematodes were exposed to 4 μM CN^- in presence of **1** (20 μM), a trace amount of fluorescence was mainly observed in the peripheral region (Figure 4B). However, when the concentration of CN^- was increased to 100 μM under same condition, strong fluorescence was observed throughout the whole body of the nematode (Fig. 4C). Noticeably, compared to **1**, such *in-vivo* imaging for cyanide detection was relatively less sensitive when nematodes were exposed to allied *in-situ* complex, followed by CN^- addition (Fig.4D-F, S11, ESI†). Furthermore, assessment of toxicity in the form of lethality assays[11] with the wild type worms revealed no effect on the survival of the worms even in higher 1 mM doses of the sensor **1** as well as the **HHMB** (Table S4, ESI†). Thus our probe **1** deserves a novel status for *in-vivo* imaging under physiological conditions.

In conclusion, we have synthesized a nontoxic fluorescent chemosensor $[Cu(BP)\mathbf{HMB}]_2(ClO_4)_2$ (**1**), that remains in monomeric form (**1a**) in aqueous medium. The choice of $Cu(BP)(ClO_4)_2$ instead of $Cu(ClO_4)_2$ for *in-situ* complex generation is justified. However, the superior selectivity, sensitivity and bio-applicability of **1** for sensing CN^- in 100% water under physiological conditions compared to allied *in-situ* complexes renders novelty and to the best of our knowledge, no such analytical comparison was reported so far. Below μM LOD and rapid ratiometric response of the sensor are also advantageous. For CN^- sensing fluorescence "*off-on*" mechanism, we report the generation of $[Cu(BP)(CN)_x]^{n-}$ species for the first time during the displacement approach.

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Graphic Abstract

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