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A new selective turn-on fluorogenic dipodal-cobalt(II) ensemble probe for nitrite ion detection and live cell imaging

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A new dipodal-triazole-Co^{2+}-ensemble with a ligand containing quinoline moiety was designed and synthesized as a highly sensitive fluorescent sensor for nitrite in aqueous media. Based on *in situ*-formed Co^{2+}–**DTQ** (Co^{2+} chelated dipodal of triazole-quinoline) ensembles having the specific binding affinity for nitrite anions over other anions, this sensory system allows rapid recognition and quantitative detection of nitrite in neutral aqueous media in an 'off-on' fashion. To confirm the suitability of **DTQ** for biological applications, we also employed it for the fluorescence detection of the changes of intracellular NO_2^- in three different species of cultured cells in the presence of nitrite ions.

The nitrite ion is an important intermediate in the biological nitrogen cycle and is widely present in the environment[1]. Nitrites are usually used as a food preservative agent[2] of meat and fish and which are also essential nutrients for the growth of plants.[3] On the other hand, nitrite can interact with amines and proteins for producing carcinogenic N-nitrosamines,[4] which enhances the possibility of gastric cancer and deformities. Water is another major accessible source through which the safety of human health is threatened by dangerous levels of nitrite (or nitrate).[5] A number of medical issues are believed to be associated with nitrite intake. One typical example is the infant methemoglobinemia, also known as "blue baby syndrome"[6] due to passage of nitrite into the blood stream, which results in the irreversible conversion of hemoglobin to metahemoglobin with oxygen uptake and transportation compromised. Therefore, it is of significant importance to detect nitrite ion in water, food and agricultural products. The World Health Organization (WHO) recommends a maximum limit of nitrite ion in drinking water to be 65 μM.[7]

Nitrite is known to be chemically unstable in the environment. Owing to this reactivity, a rapid detection procedure is preferable, especially for on-site analysis. Many analytical methods based on different principles, including UV-vis absorbance.[8,9] Electrochemistry (such as conductimetry and amperometry),[10,11] fluorescence,[12] and chemiluminescence (CL),[13-15] have been developed. UV-vis absorbance determination is usually based on diazotization of aromatic amine using acidified nitrite. A highly colored azo chromophore is formed using a subsequent coupling reaction. The process requires close control of coupling conditions.[16] Among all of the techniques, spectrofluorimetric protocol is widely used because of its simplicity, sensitivity and low cost. A number of different fluorescent reagents have been developed, such as 5-aminofluorescein,[17] 4-hydroxycoumarin,[18] acetaminophen,[19] safranine,[20] rhodamine 110,[21] 2,3-diaminonaphthalene (DAN),[22] carbon dot[23] and so on. There are a significant number of fluorescent sensors for metal ions and small biomolecules in aqueous media. However, bioimaging studies of fluorescent chemosensors toward anions have been rarely reported, probably because of low solubility in aqueous media, low selectivity, and low sensitivity.[24] The development of fluorescent sensors for anions in aqueous media is a challenging task owing to the strong hydration nature of anions and the competition of water for the hydrogen bonding sites, which weakens the interactions of the sensors with the target anions.[25] One way to tackle this hurdle is by employing the displacement method,[26] in which the sensor-ligand-metal ion "ensemble" is nonfluorescent due to metal-ion-induced fluorescence quenching. However, the addition of anions may release the sensor ligand into the solution with revival of fluorescence.

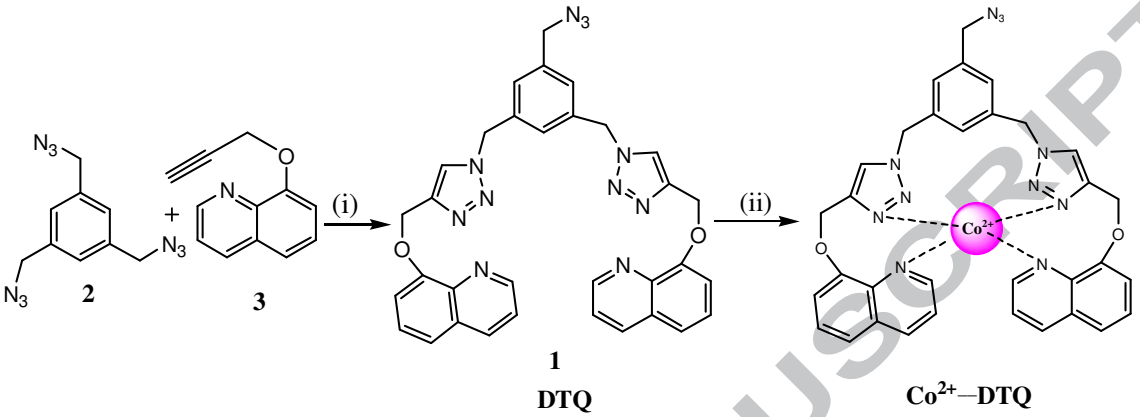
Recently, a few fluorescent chemosensors for nitrite anions have been constructed,[27] but all of them have metal reduction without fluorophore displacement or sensing through the nitrous acid specific diazotization of aromatic amine and subsequent coupling reaction with suitable aromatic reagent to yield a highly colored azo dye, the intensity of which is related to the original nitrite concentration.[28] The classic Griess reaction also used for the colorimetric detection of nitrite with Au NPs probe.[29] Despite the significant development in this domain, its ensemble version and bioimaging studies has never been explored yet for nitrite detection due to its structural simplicity. New methods have been of keen interest in molecular recognition research. Furthermore, the development of metal-based probes to fluorescent

imaging of nitrite in live cells were not satisfactory. Therefore, more nitrite ion sensors with high sensitivity and good biocompatibility are still under investigation.

By incorporating a quinoline moiety as the fluorescence active unit, we synthesized a new Co^{2+}-complex fluorescence probe Co^{2+}–**DTQ** for detection of nitrite in organo-aqueous media (Scheme 1). Such detection may be achievable when Co^{2+} chelated dipodal of triazole-quinoline (Co^{2+}–**DTQ**) moiety is generated followed by demetalization to generate fluorescence 'turn on'. Based on these results, we designed a probe for nitrite including a quinoline fluorophore and a dipodal phenyl platform receptor, which was attached via a triazole bridge. The receptor provides a rigid Co^{2+} binding site spacer between the quinoline fluorophore and chelating ligand. Quinoline and its derivatives are used extensively as fluorescence labeling reagents for their excellent photophysical properties of high fluorescence quantum yield and efficient membrane permeability.[30] The probe also provides O bridges to minimize the distance between the Co^{2+} binding site and quinoline fluorophore, which will ensure strong fluorescence quenching in the off state of Co^{2+} quinoline. At the beginning, the fluorescence of **DTQ** is quenched by coordinating to Co^{2+} ion, attributing to the paramagnetic of Co^{2+}. The Co^{2+}–**DTQ** -based nitrite probe is formed *in situ* by treating **DTQ** with one equiv of cobalt chloride. After addition of nitrite solution, nonemissive Co^{2+}–**DTQ -**"ensemble" formed of a metal-nitrite adduct, releasing a fluorophore that was initially quenched by coordination to a paramagnetic transition-metal center by electron or energy transfer.[31] Fluorophores can bind the metal center as axial ligands. Introduction of nitrite causes their displacement with concomitant fluorescence turn on. Changes in [NO_2^-] under physiological conditions will exhibit "turn-on" type fluorogenic behavior, which can be detected by measuring the ratio of fluorescence intensity with good sensitivity and selectivity. It was also expected to exhibit high sensitivity for the biological imaging of nitrite ions in living cells.

The target compound **DTQ** was readily synthesized in main two steps as shown in Scheme 1. The starting triazide-compound **2** was prepared according to the literature.[32] Reaction of 8-hydroxyquinoline with propargylbromide in dry acetone and K_2CO_3 under a nitrogen atmosphere gave compound **3**. The key intermediate, compound **2**, was then treated with compound **3** in the presence of a copper (I)-mediated click cycloaddition[33] reaction in 1:1 THF : H_2O solvent mixture to afford product **DTQ** (**1**) in 65% yield. All the new compounds

were well characterized by ^{1}H NMR, ^{13}C NMR, and mass spectroscopy (See supplementary data Figs. S1-S5).The chelated ensemble Co^{2+}–**DTQ** was easily prepared by addition of $CoCl_2$ to **DTQ** (See supplementary data Fig. S6a).



Reagent and conditions: (i) Na-ascorbate, $CuSO_4.5H_2O$, THF, H_2O, rt. 16h. (ii) $CoCl_2$

Scheme1. Synthesis of Dipodal-Triazole-Quinoline (**DTQ**) and ensamble of Co^{2+}–**DTQ.**

With compound **DTQ** in hand, we examined its optical properties in the absence or presence of various heavy and transition metal species. The free compound **DTQ** exhibited an absorption band at around 261 nm in pH 7.4 HEPES buffer (30 mM, pH 7.4, containing 70% CH_3CN).

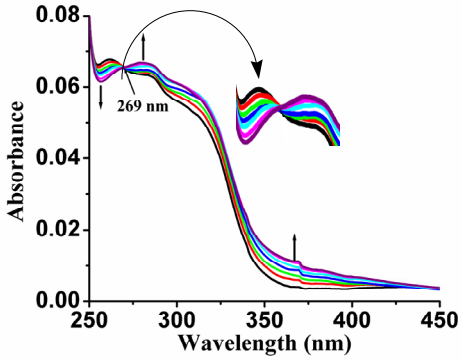


Figure 1. Absorption titration spectra of **DTQ** (c = 1.0×10^{-5} M) upon addition of Co^{2+} ions (0-2 equiv) in pH 7.4 HEPES buffer (30 mM, pH 7.4, containing 70% CH_3CN).

Gradual addition of cobalt perchlorate to a solution of **DTQ** in HEPES buffer, induces a 19 nm red shift of the absorption from 261 to 280 nm along with a shoulder peak at 295 nm (Fig.1). A clear isosbestic point was observed at 269 nm, which is consistent with the

presence of only two species, free ligand, and Co^{2+}–ligand ensemble. No marked changes in the absorption profiles were noted upon the addition of Al^{3+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, Mn^{2+}, Fe^{3+}, Ni^{2+}, and Cu^{2+}, respectively. These results clearly suggested that the metal complexation of **DTQ** show a great preference for cobalt ion over other cations. The binding constant was determined to be $(1.25\pm0.25)\times10^5$ M^{-1} using linear regression Benesi–Hildebrand equation analysis[34] (See supplementary data Fig. S6b).

The corresponding fluorescence spectra have been measured for the **DTQ**'s solution (1.0 μM, pH 7.4, containing 70% CH_3CN) in the absence and presence of various metal ions. The free ligand **DTQ** is fluorescent with an emission peak at around 395 nm (Φ_{fl}=0.71).[35] From the Figure 2a and its inset, it can be seen that its intense emission peak at 395 nm is nearly quenched (9.5 fold) upon addition of 1.2 equiv of Co^{2+}, concomitant with a new weak band at around 325 nm and a distinct isoemissive point at 344 nm was observed. This could be attributed to the photoinduced electron transfer (PET)[36] from the quinoline fluorophore to the chelated Co^{2+} and probably due to the paramagnetic nature of the Co^{2+} ion.[37]

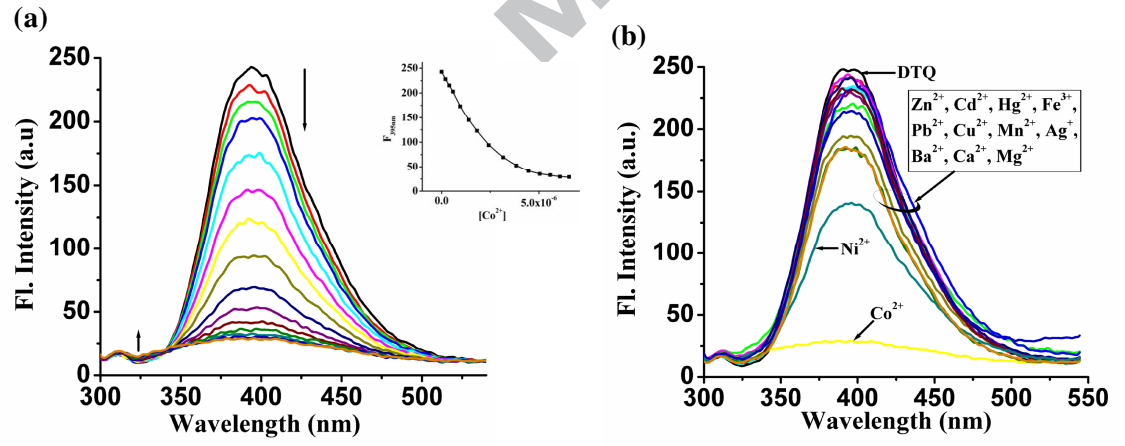


Figure 2. (a) Fluorescence titration of **DTQ** (c = 1.0×10^{-6} M) in HEPES buffer (50mM, pH 7.4) in the presence of different amounts of cobalt perchlorate (λ_{ex}=285 nm). The inset shows the fluorescence intensity changes at 395 nm of compound **DTQ** (10 μM) with the amount of Co^{2+} ions. (b) Fluorescence emission changes in **DTQ** (10 μM), upon addition of different metal ions (1.0 equiv) in 70% CH_3CN-water at pH 7.4 (λ_{ex}=285 nm).

However, addition of heavy and transition metal ions caused fluorescence quenching to a different extent (See supplementary data Fig. S7), which is shown in Figure 2b. In particular,

the fluorescence of **DTQ** is almost completely quenched by Co^{2+} ions. Thus, we decided to further evaluate the Co^{2+} -responsive nature of compound **DTQ**.

In good agreement with this finding, the Job plot[38] also shows the formation of a 1:1 bonding mode between **DTQ** and Co^{2+} ions (Fig. 3). The association constant of **DTQ** with Co^{2+} was determined to be $(1.49\pm0.51)\times10^5$ M^{-1} on the basis of the fluorescence titration experiments (See supplementary data Fig. S8). The 1:1 binding model of $Co^{2+}$and **DTQ** can be further confirmed by mass spectra. The ESI mass spectrum of complex **DTQ**–Co^{2+} has a major peak with m/z of 670.018 [Co^{2+}–**DTQ**], which corresponds to 1:1 complex (See supplementary data Fig. S9).

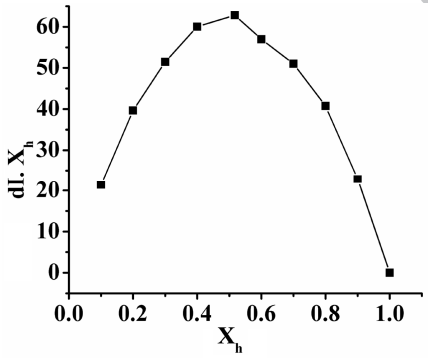


Figure 3. Fluorescence titration Job plot of the receptor **DTQ** with Co^{2+}, where [G] = [H] = 1.0×10^{-6} M in HEPES buffer (CH_3CN : H_2O = 7:3, v/v, 50mM, pH 7.4).

Based on the above finding results of titration experiments and mass spectrum of Co^{2+}–**DTQ** complex, the chelated complex Co^{2+}–**DTQ** was easily prepared by addition of cobalt chloride to **DTQ** in the solution of HEPES buffer (50 mM, pH 7.4, containing 70% CH_3CN). We found that Co^{2+}–**DTQ** ensemble is initially nonfluorescent (Φ_{fl}=0.05), the fluorescence intensity increased steadily with increasing $NaNO_2$ concentration (Fig. 4a) until it reached a plateau at 1.0 equiv $NaNO_2$, which corresponded to a 9.5-fold increase in fluoresence instensity compared to the blank [NO_2^-]. This indicates that complete displacement of Co^{2+} from Co^{2+}–**DTQ** occurred with one equiv $NaNO_2$ addition. The fluorescence intensities at 395 nm have an excellent linear relationship with the concentrations of [NO_2^-] anions (Fig. 4a, inset). To test this idea, the absorption spectra of Co^{2+}–**DTQ** ensemble were recorded by gradual addition of nitrite to a solution of Co^{2+}–**DTQ** in HEPES buffer. As shown in Figure 4b the absorption spectra, which is essentially identical with the maximal wavelength of the

absorption peak of the free **DTQ**, indicating that addition of nitrite anions to the ensemble resulted in the release of the free **DTQ**. Consistent with this observation, treatment of nitrite caused a significant fluorescence turn-on response at 395 nm (Fig. 4a), and up to a 9.5-fold fluorescence enhancement (Φ_{fl}=0.75) was observed.

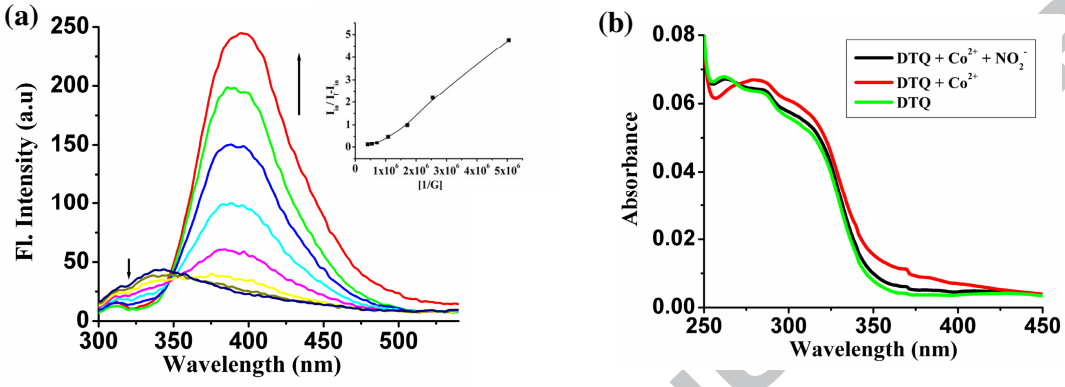


Figure 4. (a) Fluorescence spectra of the Co^{2+}–**DTQ** ensemble (1 μM) in pH 7.4 HEPES buffer (25 mM, pH 7.4, containing CH_3CN : H_2O = 7:3, v/v) in the presence of NO_2^- anions (0-1 equiv). The inset shows the fluorescence intensity changes at 395 nm of the ensemble (10 μM) in the presence of increasing nitrite concentrations (0-1 equiv). (b) The absorption spectra of the Co^{2+}–**DTQ** ensemble (10μM) in pH 7.4 HEPES buffer (30 mM, pH 7.4, containing CH_3CN : H_2O = 7:3, v/v) in the presence of nitrite anions.

To identify whether the Co^{2+}–**DTQ** ensemble was highly sensitive to NO_2^-, we examined a wide array of possible competitive reactive oxygen (ROS) and nitrogen species (RNS) and other analytes in organo-aqueous media (25 mM, pH 7.4, containing 70% CH_3CN) at up to 100-fold excess. As shown in Figure 5a, 100 equiv of F^-, Cl^-, Br^-, I^-, NO_3^-, N_3^-, SCN^-, HPO_4^{2-}, SH^-, PPi and 10 equiv of CN^-could not induce any marked fluorescence enhancement. By sharp contrast, one equiv of nitrite elicited a large fluorescence enhancement in the region of 395 nm. Competitive experiments were conducted with one equiv $NaNO_2$ and 100 equiv various ROS and RNS. The fluorescence intensity did not vary much in comparison with that produced by NO_2^-+Co^{2+}–**DTQ** (Fig. 5b). These results indicate that Co^{2+}–**DTQ** displays high selectivity toward NO_2^- over other ROS and RNS species.

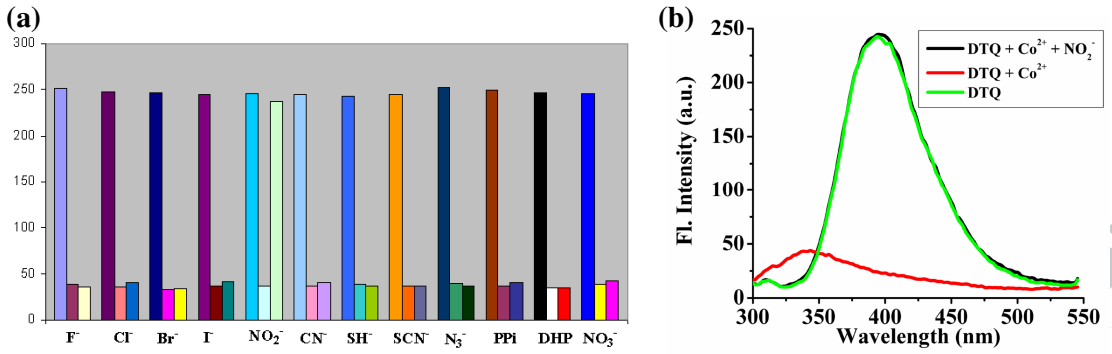
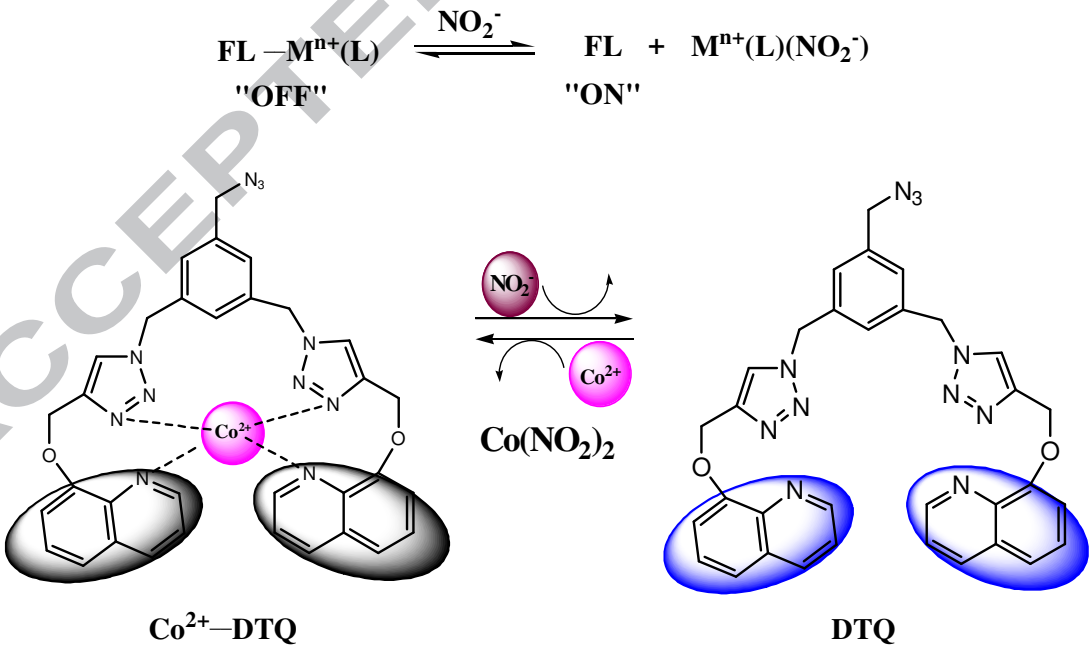


Figure 5. (a) Bars represent the fluorescence responses to various ROS and RNS species at 395 nm. The left hand bars represent only **DTQ**, middle bars Co^{2+}–**DTQ** ensemble and right hand bars Co^{2+}–**DTQ** ensemble + representative anions respectively. Spectra were acquired in HEPES buffer (25 mM, pH 7.4, containing CH_3CN : H_2O = 7:3, v/v). (b) Normalized fluorescence spectra of **DTQ** (green), **DTQ** +Co^{2+}(red), and Co^{2+}–**DTQ** ensemble+NO_2^- (black).

To gain insight into the sensing mechanism, we decided to study the sensing process by ^{1}HNMR spectra in presence of nitrite ions. The product of Co^{2+}–**DTQ** + nitrite anions was isolated by a silica gel column and was then subjected to ^{1}H NMR analysis. The ^{1}H NMR and mass spectra of the resulting product are essentially identical to that of free sensor **DTQ**.



Scheme 2. Proposed displacement mechanism for sensing of NO_2^- anions.

Thus, the studies of NMR, mass spectrometry, absorption spectrometry, and fluorescence spectrometry indicate that the sensor functions by the displacement mechanism (Scheme 2).

Acid-base fluorescence titrations revealed that the fluorescence intensity of Co^{2+}–**DTQ** ensemble was unaffected by pH values between 6.08 and 9.41 (excitation at 285 nm). This suggests that the probe would work well at physiological pH, although the fluorescence intensity of Co^{2+}–**DTQ** ensemble was quenched slightly at pH <6.0 (See supplementary data Figs. S10).

To further demonstrate the practical biological application of the sensor **DTQ**, fluorescence imaging experiments were carried out in living cells. Here we used three different sets of living cells for imaging purpose. *Candida albicans* (Set 1), *Bacillus subtilis* (Gram-positive, rod shaped bacteria) (Set 2) and *Pollen* (Set 3) cells were first incubated with 20 μM of sensor **DTQ** (in the MEM (modified Eagle's medium) culture medium containing 1:1 v/v water-ethanol) for 30 min at 37°C in three different cover glass bottom dish, and then washed with PBS (containing 2.0% methanol) (0.1M, pH = 7.4) three times to remove excess of **DTQ**.

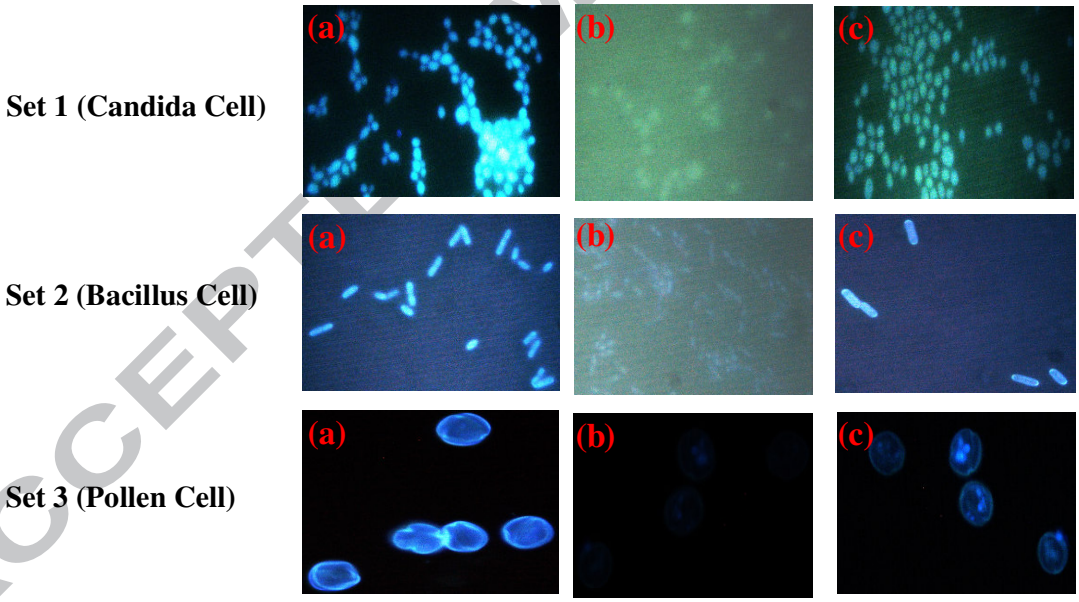


Figure 6. Comparison of three sets of cells by fluorescence microscopic experiment (a) images of *Candida albicans*, *Bacillus subtilis* and *Pollen* cells + **DTQ**, (b) images of *Candida albicans*, *Bacillus subtilis* and *Pollen* cells + **DTQ** + Co^{2+} (c) images of *Candida albicans*, *Bacillus subtilis* and *Pollen* cells + **DTQ** + Co^{2+} + NO_2^- (5 μM).

Figure 6a showed that when all three types of cells were treated with sensor **DTQ** only, bright fluorescence were detected. Then, the treated cells were incubated with 30 μM $CoCl_2$ and the fluorescence signal was monitored again, which produced very faint intracellular fluorescence (Fig. 6b). The fluorescence intensities were further observed inside of the cells, after the $NaNO_2$ ($c = 1.2\times 10^{-6}$ M) treatment. The bright field transmission images of these cells in Figure 6c is exactly the same as the fluorescence image in Figure 6a, confirming that the imaged fluorescence is intracellular, instead of extracellular. Appearance of the blue fluorescence confirmed the displacement of Co^{2+} from Co^{2+}–**DTQ** ensemble and free **DTQ** was adsorbed within the bacterial cells. The resluts suggest that sensor **DTQ** is cell membrane permeable[39] and can also be used for intracellular imaging of NO_2^- in living cells and potentially *in vivo*.

In this contribution, we have synthesized a cobalt (II) ensemble Co^{2+}–**DTQ** with a triazole N-donor ligands having a pendant quinoline moiety as a fluorophore probe for NO_2^- sensing and bioimaging. **DTQ** showed excellent selectivity for Co^{2+} over competing metal ions even at low concentrations. This ensemble probe Co^{2+}–**DTQ** was NO_2^- specific and provided high selectivity over other ROS and RNS species. Fluorescence imaging shows that Co^{2+}–**DTQ** could be used for detection of changes in NO_2^- levels in living cells with low cytotoxicity. Furthermore, the confocal fluorescence images of *Candida albicans, Bacillus subtilis* and *Pollen* cells suggested that Co^{2+}–**DTQ** was successfully applied for monitoring intracellular NO_2^-.

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Supporting Information Available:

Experimental details, spectral data, NMR spectra, mass spectra, binding studies.

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39. *MTT {3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide} assay on experimental cells were done in presence and absence of* ***DTQ*** *and* Co^{2+}*-salt and the result indicates that mitochondrial succinate dehydrogenase reduces yellow coloured MTT into an insoluble dark purple coloured formazan. Since reduction of MTT can only occur in metabolically active cells (due to presence of active dehydrogenase) the level of activity is a measure of the viability of the cells.*

Graphical Abstract

A new selective turn-on fluorogenic dipodal-cobalt(II) ensemble probe for nitrite ion detection and live cell imaging

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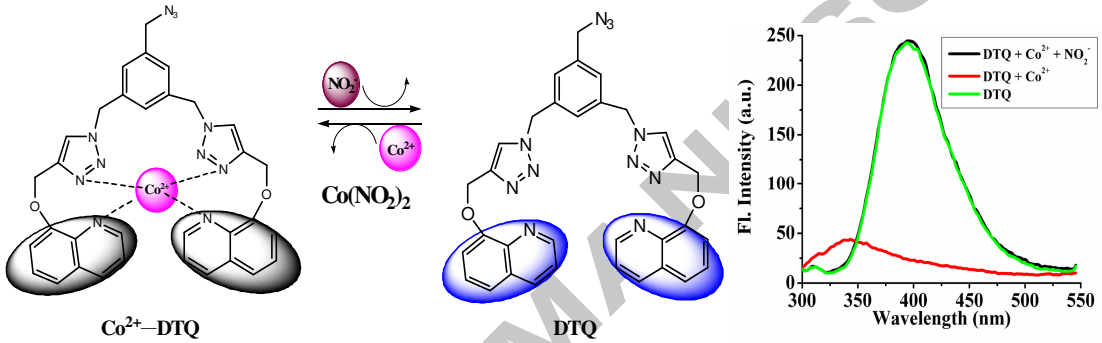

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