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A reaction based turn-on type fluorogenic and chromogenic probe for the detection of trace amount of nitrite in water

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ABSTRACT

A turn-on fluorescent probe for the detection of nitrite ion in water is developed based on diazotization reaction of the amino group of the probe in an acidic solution (pH 1). The probe responds selectively to nitrite ion over various other anions with a turn-on type fluorogenic change from colorless to orange by the formation of rhodamine B via an analyte triggered fragmentation process. The fluorescence titration is complete within 1 h with 1 equivalent of nitrite ion. The probe is highly efficient, cost-effective and shows a detection limit of 4.6 ppb.

1. Introduction

An uncontrolled increase in the concentration of nitrite in groundwater, rivers and lakes is a growing threat to public health and the environment [1]. Nitrite is used as a fertilizing agent and preservative of perishable foods. It is also known to play a key physiological role in signaling, blood flow regulation and hypoxic nitric oxide homeostasis [2]. Monitoring of nitrite level in drinking water and food stuffs is of great importance as it reacts with dietary components in the stomach to produce carcinogenic nitrosamines [3]. Another potential danger with the nitrite ions is its ability to convert oxyhemoglobin into methemoglobin when present in the bloodstream and thereby interfering with oxygen transport in the blood [4]. Epidemiologic studies have revealed that elevated concentration of nitrate and nitrite ions in drinking water leads to a number of medical issues like spontaneous abortions, premature birth, intrauterine growth restriction and birth defects of the central nervous system [5–9]. The permitted level of nitrite content in water is just 1 ppm as recommended by the US Environmental Protection Agency (EPA) [10,11]. Therefore, sensitive and selective methods are required for the determination of nitrite.

A number of techniques have been developed for the detection of nitrite ions, based on organic chromophores [12–14], electrochemical detection [15], ion chromatography [16,17] and others [18,19]. Many of these approaches use sophisticated instruments and are expensive and time consuming. In addition, some of these are not adequately sensitive or selective for the determination of trace amount of nitrite ions. Therefore, they cannot be used for real-time and on-site measurement of nitrite. In recent time, a few sensitive colorimetric nitrite sensors using plasmon resonance based gold nanorods [20] and gold nanoparticle probes [21], and a reaction-based probe [22] have been reported. However, probes based on fluorometry [23–26] attract special attention owing to their favorable features such as operational simplicity and cost-effectiveness, in addition to the high sensitivity and selectivity. In particular, reaction-based fluorometric probes, called as chemodosimeters or chemoreactants, which involve target analyte induced fast and irreversible chemical reactions coupled with instant signal transduction, are more efficient in terms of sensitivity and selectivity than chemical probes based on non-covalent interactions in most cases [27]. Application of fluorophore based chemodosimeters in the detection of nitrite ion is rare [23,24]. The reported probes follow tedious procedure and suffer from poor detection limit. We report herein a rhodamine based "turn-on" type fluorogenic chemodosimeter, **1**, which detects trace amount of nitrite ions in water as low as 4.6 ppb. In addition, the naked eye detection level of this probe is well below the range of the EPA recommended MCL for nitrite.

2. Experimental

2.1. Apparatus

NMR spectra were recorded on Bruker AV300 NMR spectrometer. Mass spectra were obtained from Waters Q-TOF micro

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mass spectrometer (ESI^+) and Agilent 6400B LC–MS (ESI^+). Fluorescence spectra were taken on a JASCO FP-6300 spectrofuorometer, the slit width was 2.5 nm for both excitation and emission. Absorption spectra were recorded on a JASCO V570 UV/Vis/NIR spectrophotometer.

2.2. Reagents

Rhodamine B hydrochloride was purchased from Sigma Aldrich and used as received. All other chemicals were obtained from different commercial suppliers and were used without further purification.

2.3. General procedure

Chloroform was distilled from P_2O_5 and acetonitrile was distilled from CaH_2 before use. The reactions were monitored by thin layer chromatography (TLC) carried out on 0.25-mm silica gel plates (60F-254) using UV light (254 or 365 nm) or naked eye for visualization. Probe **1** was dissolved in 95% aqueous ethanol to make $2.0x10^{-3}$ M stock solution, which was diluted to required concentration for measurement. A $1.0x10^{-3}$ M stock solution of $NaNO_2$ was prepared in deionized water (MilliQ, 18 Ω) and other standard solutions of $NaNO_2$ were prepared by further dilution. For study of the effect of different anions and the competitive study of nitrite with other anions, stock solutions (5 mL, 1 mM) were prepared by dissolving $Pb(NO_3)_2$, K_2CO_3, CH_3COONa, NaI, Na_2SO_4, $(Bu)_4N^+Br^-$ and $Na_2S_2O_3$ in deionized water (MilliQ, 18 Ω). All solutions are subjected to filtration through 0.22 μm syringe filter in order to avoid any interference by any particulate matter in fluorescence measurement.

2.4. Synthesis of probe *1*

To a solution of rhodamine B, HCl (100 mg, 0.21 mmol) in dry chloroform (4.0 mL) at room temperature and phosphorus oxychloride (0.058 mL 0.63 mmol) was added dropwise over a period of 5 min. After being refluxed for 4 h, the reaction mixture was cooled and concentrated under vacuum to get rhodamine B acid chloride. This acid chloride was dissolved in dry acetonitrile (2.0 mL) and added dropwise to a solution of o-phenylenediamine (103 mg, 0.95 mmol) in dry acetonitrile (0.6 mL) containing triethylamine (0.8 mL). After stirring for 10 h at room temperature, the mixture was concentrated under vacuum and the crude product was purified by column chromatography (ethyl acetate–petroleum ether 60–80, 20:80) to give compound **1** as white solid in 78% yield; mp 178–179 °C.

^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 1.14 (*t*, J=7.2 Hz, 12H); 3.25–3.39 (*d*, J=7.2 Hz, 8H); 3.41 (bs, 2H), 6.09 (dd, J_1=1.0 Hz, J_2=7.5 Hz, 1H); 6.26 (bs, 2H); 6.30 (*d*, J=10 Hz, 2H); 6.41 (dt, J_1=1.0 Hz, J_2=7.5 Hz, 1H); 6.55 (dd, J_1=1.0 Hz, J_2=7.5 Hz, 1H); 6.64 (d, J=10 Hz, 2H); 6.95 (dt, J_1=1.0 Hz, J_2=7.5 Hz, 1H); 7.23–7.26 (m, 1H); 7.53–7.57 (m, 2H); 8.01–8.04 (m, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 12.6; 44.4; 68.1; 98.0; 106.9; 108.0; 117.0; 118.2; 122.2; 123.5; 124.3; 128.4; 128.7; 128.8; 128.9; 132.0; 132.7; 144.5; 148.9; 152.4; 154.0; 166.5. ESI–MS (*m*/*z*): 533.4 ($[M+H]^+$), 555.4 ($[M+Na]^+$); calculated 532.2.

3. Result and discussions

3.1. Synthesis and spectral characterization of probe *1*

The colorless and nonfluorescent probe **1** was first synthesized in a "one-step" synthetic procedure as described by Zheng et al. [28] with minor modification from rhodamine B in high yield. The free acid group of rhodamine B HCl was converted to acid chloride by refluxing with phosphorus oxychloride and the resulting crude mixture in acetonitrile was treated with o-phenylenediamine to produce probe **1** in 78% yield. The compound was characterized by ^1H NMR, ^{13}C NMR and ESI–MS, which is in complete agreement with the reported value.

3.2. Study on pH sensitivity and nitrite sensing condition of probe *1*

The function of probe **1** as nitrite detector is based on the diazotization of the amino group of the probe. As the diazotization process requires strong acidic condition, a study on pH dependency of probe **1** was carried out to ensure its stability at low pH. The fluorescence intensity of various solutions of probe **1** within a pH range of 1–9 was measured after 1 h of the addition of the probe at 0–5 °C. Each of the solutions exhibited little or no fluorescence revealing that the spirocyclic form is stable within a wide range of pH at the prevailing condition of diazotization reaction (Fig. 1). Next, the fluorescence intensity of solutions containing probe **1** at pH 1 was measured against time both at low temperature (0–5 °C) and room temperature. To our delight, it was observed that the probe **1** can sustain strong acidic condition (pH 1) at low temperature for several hours with negligible change in fluorescence intensity with respect to a neutral aqueous solution of the probe (Fig. 2) and without developing any visible color in the solution. However, the same acidic solution of probe **1** at room temperature gradually showed increase in fluorescence intensity at λ_{max} 598 nm and development of purple color. The saturation point of fluorescence intensity was reached after exposure of probe **1** at pH 1 for 24 h. From this study we presume that spirocyclic lactam form of probe **1** remains in equilibrium with the ring-opened amide form of probe **1** under acidic condition (Scheme 1) [24]. The presence of ring-opened amide form is negligible even at low pH (viz. pH 1) when the probe solution is kept at 0–5 °C but gradually increases in number at room temperature.

The most suitable condition of diazotization reaction was determined by measuring fluorescence intensity of various solutions at different pH containing 1 equivalent of nitrite ions. Thus, 1 equivalent of $NaNO_2$ was added to each of the solutions within a pH range of 1–9 at 0–5 °C and their fluorescence intensity was recorded after 1 h. This study revealed that the most suitable pH for the sensing reaction is 1 (Fig. 1). The reaction rate gets slower with the decrease in acid strength up to pH 4. As expected, probe **1** does not work at near to neutral or basic pH. Low temperature, a requirement of conventional diazotization reaction, was maintained for all fluorometric studies to avoid any side reaction.

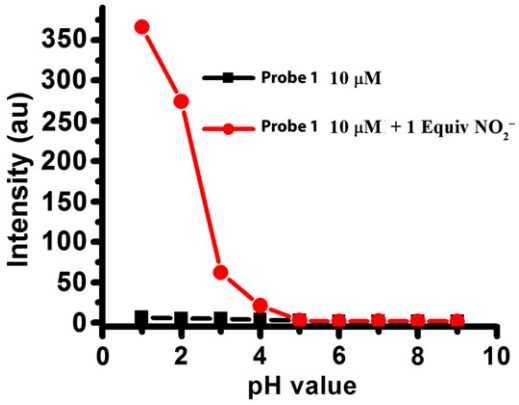


Fig. 1. Fluorescence response of probe **1** (10 μM) after 60 min with and without NO_2^- (10 μM, 1 equiv) in different pH buffer (pH 1–9) at 0–5 °C (excitation at 525 nm).

3.3. Mechanistic study of nitrite sensing by probe **1** and spectral features

Probe **1** detects nitrite ions with instantaneous development of fluorescence peak by the production of highly fluorescent rhodamine B via the diazotization of the amino group of the probe, followed by intra-molecular rearrangement and fragmentation. To establish this fact a time-dependent fluorescence measurement of a 1:1 mixture of probe **1** and $NaNO_2$ in 0.1 N HCl solution at 0–5 °C was carried out. It showed that the fluorescence intensity reaches saturation after 60 min (Fig. 3). The solution shows an intense pink color and a bright orange fluorescence. In the emission spectra, an intense emission band initially appears at λ_{max}=603 nm, which undergoes hypsochromic shift to λ_{max}=585 nm with time (Fig. 3) [28]. Compound **1** is presumed to follow the proposed mechanistic pathway of Zheng et al. and thereby, produces a temporary fluorescent species, rhodamine B acylbenzotriazole (compound **2**) (Scheme 2). As compound **2** is extremely prone to hydrolysis, it quickly dissociates into rhodamine B and benzotriazole. Although compound **2** was not isolable from aqueous solution, formation of this unstable species is supported by the appearance of fluorescence peak first at a higher wavelength (at λ_{max}=603 nm) followed by a blue shift of ~18 nm to show characteristic peak of rhodamine B (at λ_{max}=585 nm). This is in well agreement with the difference in emission wavelength between rhodamine B tertiary amide and rhodamine B [29]. The formation of compound **2** was further established by the appearance of its molecular ion peak in the ESI–MS spectrum obtained from a sample reaction mixture after allowing the reaction to proceed for just 10 min (see supporting information, Fig. S1). Both rhodamine B and benzotriazole were isolated in good yield from a separate reaction of compound **1** with sodium nitrite in 0.1 N HCl solution and characterized by spectral analysis.

3.4. Spectrofluorometric and spectrophotometric titrations of probe **1**

The fluorometric titration of probe **1** with NO_2^- was done by measuring fluorescence emission of the acidic solution of probe upon gradual addition of 0–2 equivalent of NO_2^-. It showed saturation in the emission intensity at the equivalence point (Fig. 4). It reflects that the intra-molecular rearrangement is a much quicker process than the attack of any external species (e.g. water) to the diazotized aromatic ring. In a similar study, chromogenic response of probe **1** towards NO_2^- was checked by measuring absorbance of the acidic solution of probe upon gradual addition of 0–2 equivalent of NO_2^-. A strong absorbance peak at λ_{max}=558 nm was developed and intensified with increasing concentration of nitrite ions in solution (Fig. 5). Both the studies revealed that the reaction goes to completion after addition of 1 equivalent of nitrite ions.

3.5. Selectivity of NO_2^- over other anions

The fluorescence sensing selectivity of this system for NO_2^- was examined. Under the same conditions as used above for NO_2^-, we tested the fluorescence responses of probe **1** towards other anions such as CO_3^{2-}, SO_4^{2-}, I^-, CH_3COO^-, $S_2O_3^{2-}$, NO_3^- and Br^-. As depicted in Fig. 6, probe **1** showed essentially no response toward other anions (for bar graph see Fig. S2 of supporting information). The specificity is a consequence of the fact that diazotization of the amine group of probe **1** is possible only by nitrite ions.

Achieving high selectivity towards the desired analyte amongst many other competitive species coexisting in the sample is an important feature to evaluate the performance of a fluorescence chemodosimeter. Therefore, several solutions were prepared by adding one equivalent each of $NaNO_2$ and other possible interfering ions such as NO_3^-, CO_3^{2-}, SO_4^{2-}, I^-, CH_3COO^-, $S_2O_3^{2-}$ and

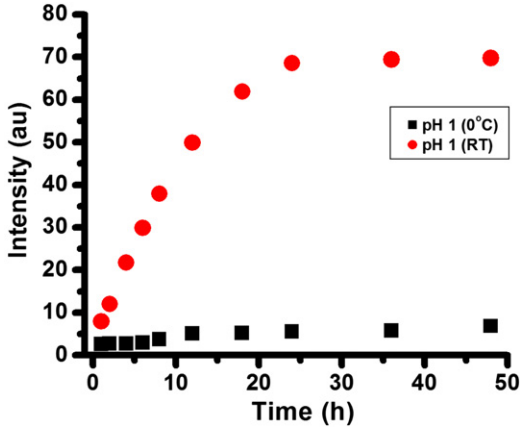


Fig. 2. A plot of fluorescence response of acidic solutions of probe **1** (10 μM in 0.1 N aqueous HCl solution) kept at 0–5 °C and room temperature against time (excitation at 525 nm). The gradual increase in fluorescence intensity with time at room temperature is due to the opening of the spirocyclic lactam ring of probe **1** to produce the ring-opened amide form.

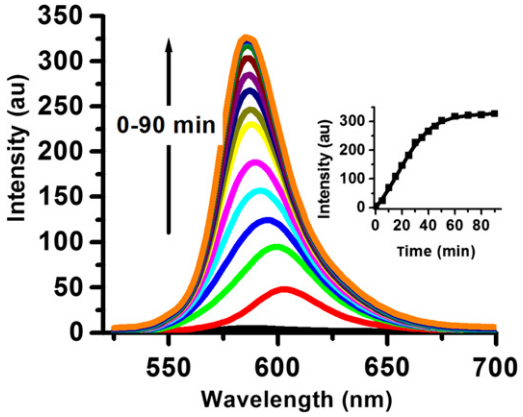
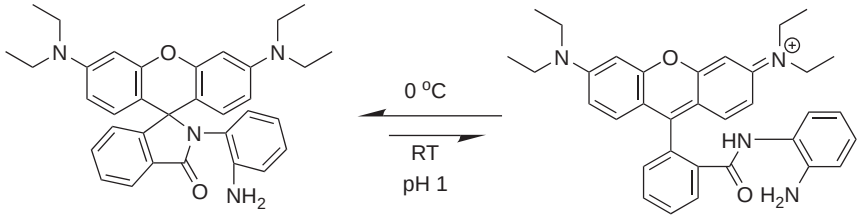
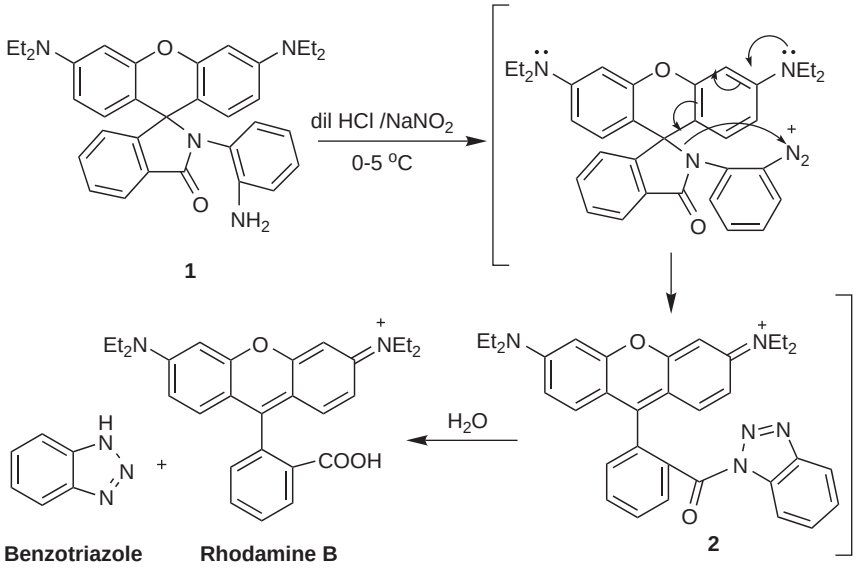


Fig. 3. Fluorescence response of probe **1** (10 μM in 0.1 N aqueous HCl solution) upon addition of 10 μM (1 equiv) of NO_2^- at 0–5 °C after each 5 min interval up to 90 min (excitation at 525 nm). Inset: fluorescence intensity vs. time plot showing saturation after 60 min.



Scheme 1. Schematic representation showing that the spirocyclic lactam form of **1** remains in equilibrium with the ring-opened amide form at pH 1.



Scheme 2. NO_2^- induced ring opening of probe **1** to produce fluorescent rhodamine B.

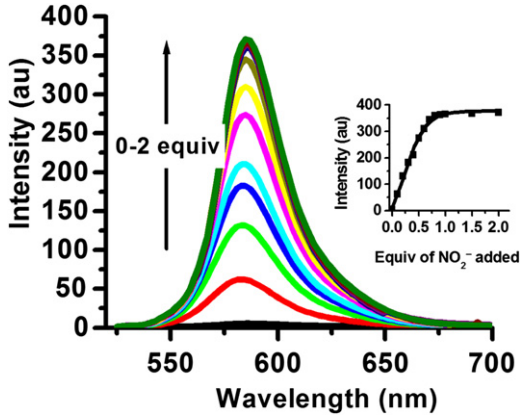


Fig. 4. Fluorescence response of **1** (10 μM after 60 min upon addition of 0–2 equiv of NO_2^- in 0.1 N HCl solution at 0–5 °C (excitation at λ_{max}=525 nm). Inset: a fluorescence intensity plot showing dependency on the equiv of nitrite ion.

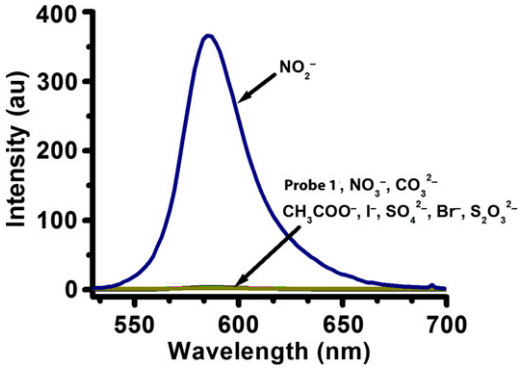


Fig. 6. Fluorescence response of probe **1** (10 μM) in the presence of 1 equiv of different anions (inorganic salts used are $Pb(NO_3)_2$, K_2CO_3, CH_3COONa, NaI, Na_2SO_4, $(Bu)_4N^+Br^-$, $Na_2S_2O_3$, respectively) in 0.1 N aqueous HCl solution at 0–5 °C after 60 min of the addition of salts (λ_{ex}=525 nm, λ_{em}=585 nm).

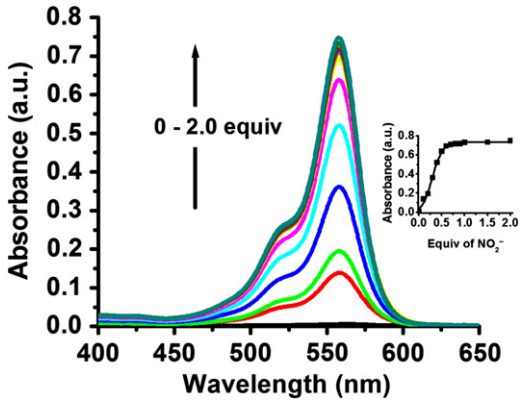


Fig. 5. Absorbance response of probe **1** (10 μM) after 60 min upon addition of 0–2 equiv of NO_2^- in 0.1 N HCl solution at 0–5 °C (excitation at 525 nm). Inset: an absorbance intensity plot showing dependency on the equiv of nitrite ion.

Br^- in 0.1 N HCl at 0–5 °C and allowed to stand for 5 min before addition of one equivalent of probe **1**, and the fluorescence intensity of the resulting solutions was measured after 1 h of incubation. As expected, the nitrite-induced fluorescence intensity of probe **1** was not much influenced by the presence of equivalent amount of most of the anions (Fig. 7). However, only a slight drop in the fluorescence intensity was observed in case of oxidizable anions like I^-, $S_2O_3^{2-}$and Br^-. Oxidizable anions, particularly iodide, are expected to interfere strongly in the detection of nitrite ions by spontaneously reacting with them under acidic condition to produce NO gas [30]. On the other hand, reactive radical species, NO does not survive in aqueous solution for long time but undergoes oxidation to produce mainly nitrite ions [31]. Considering these facts, we assume that both the reactions take place in a consecutive manner, at different extents depending on the kind of oxidizable anion present in the solution for the competition experiments, to maintain similar level of nitrite ions in solution rendering negligible drop in the fluorescence intensity. Our assumption is supported by the fact that probe **1** shows strong fluorescence emission in the presence of NO under the prevailing diazotization condition at pH 1 (see supporting information for details, Fig. S3).

3.6. Measurement of detection limit

Probe **1** responds to NO_2^- ions linearly well below the micromolar level concentration range (Fig. 8), and from that the detection limit (defined as three times standard deviation of

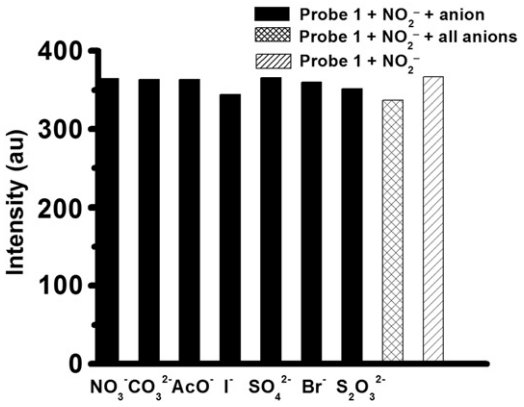


Fig. 7. Selectivity of the probe **1** towards NO_2^- amongst many other competitive species coexisting in 0.1 N aqueous HCl solution at 0–5 °C (λ_{ex}=525 nm, λ_{em}=585 nm).

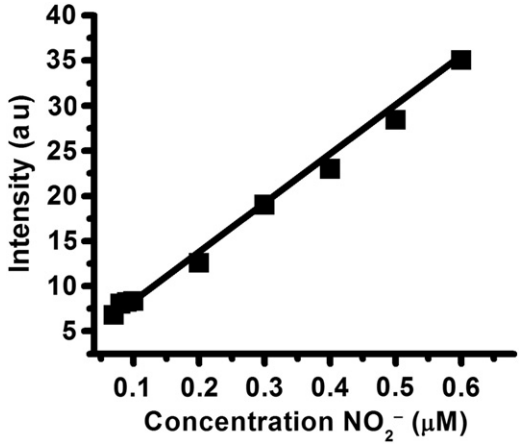


Fig. 8. A fluorescence intensity plot of probe **1** against low concentration range of NO_2^-. The straight line was obtained from 0.1 μM of NO_2^- or 4.6 ppb of NO_2^-.

probe intensity) of the probe is estimated to be 1.0×10^{-7} M of $NaNO_2$ or 4.6 ppb [NO_2^-].

3.7. Real sample analysis

Probe **1** was applied for quantitative detection of nitrite level in several real samples. The samples were collected from various sources like river, paddy field, drinking water, etc. For the determination of the concentration of NO_2^- each real sample was made appropriately acidic by the addition of conc. HCl; probe **1** was added at 0–5 °C and fluorescence intensity of the resulting solution was measured after 1 h. As shown in Fig. 9, the nitrite level of real samples was found in the range 8–40 ppb by plotting the intensity values in a standard fluorescence intensity curve. The chromogenic property of the probe was also useful for on-spot qualitative detection of nitrite level in the real samples. Each of the above solutions showed visible color change from colorless to purple.

4. Conclusions

In summary, we have developed a rhodamine-based turn-on type fluorescent probe for the detection of trace amount of nitrite ions in water. The function of the probe depends on diazotization of its amino group followed by opening of the spirocyclic ring, intra-molecular rearrangement and fragmentation to produce

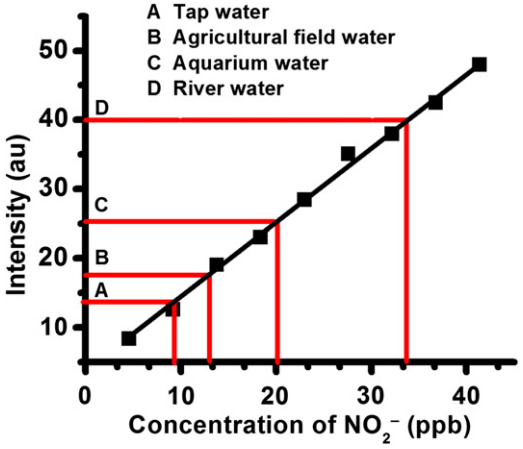


Fig. 9. (A) A plot of the intensities of different real samples on to standard fluorescence intensity curve to quantify the level of NO_2^- ions in those samples. 3 mL of each real sample was made adequately acidic by the addition of conc. HCl to make it 0.1 N and probe **1** was added at 0–5 °C and fluorescence intensity of the resulting solution was measured after 1 h (λ_{ex}=525 nm, λ_{em}=585 nm). (B) A photograph showing chromogenic response of real samples: (a) blank, (b) tap water, (c) agricultural field water, (d) aquarium water, and (e) river water. (C) A photograph showing fluorogenic response of real samples: (a) blank, (b) tap water, (c) agricultural field water, (d) aquarium water, and (e) river water.

rhodamine B. The probe is extremely sensitive and selective to nitrite ion in the presence of many other anionic species, showing a turn-on type fluorescence change. The chromogenic property of the probe can be used for on-spot qualitative detection of nitrite level in the real sample. High sensitivity and selectivity, fast reaction coupled with cost effective synthesis make this probe highly considerable for practical use.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2012.06.045.

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