Analytical Methods

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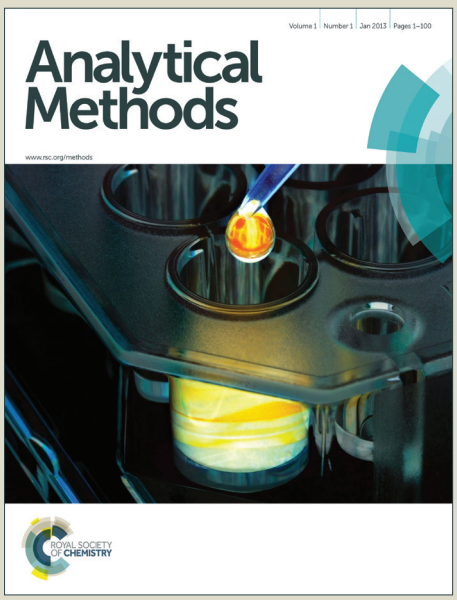

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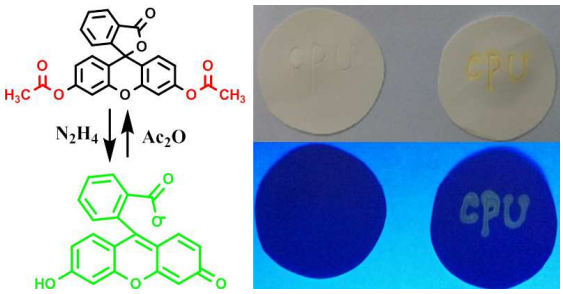

Highlights

1. Work well in aqueous solution at neutral pH
2. Excellent specificity
3. Visualization of vaporized hydrazine

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PAPER

A fluorescence "switch-on" approach to detect hydrazine in aqueous solution at neutral pH

Dian-Yun Qu,[a,1] Jin-Long Chen,[a,b,1*] Bin Di[a,b*]

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A new fluorescent probe capable of reliable detection of hydrazine under environment-friendly conditions with high specificity and sensitivity was developed. 3, 6-diacetoxyfluoran (FDA), a readily commercially available compound was explored for fluorescence "switch-on" detection of hydrazine in this work. FDA can specially hydrazinolyze and transform into fluorescein as a product in aqueous solution at neutral pH, resulting in distinct optical changes from colorless to green and "switch-on" fluorescence which allows for establishing a new method for sensitive detection of hydrazine. Under optimum conditions described in this work, the enhancement of fluorescence at 515 nm was linearly proportional to the concentrations of hydrazine ranging from 1.25 to 25.00 μM with a correlation coefficient of $R^2 = 0.9959$ and a detection limit as low as 31 nM. The relative standard deviation of twelve replicate measurement samples was 5.4 % for 12.5 μM hydrazine. The proposed method was convenient, low cost and free of complex equipment, making it possible to successfully determine hydrazine in four real samples containing distilled water, tap water, isoniazid and plasma samples. Furthermore, the hydrazine probe would be a promising candidate capable of naked-eye visualization of gaseous hydrazine by simple operations.

Introduction

Hydrazine (N_2H_4), as a highly reactive and reducing agent, has been widely applied in many fields, including chemical, pharmaceutical and agricultural industries.[1] It is famous as a rocket propellent for its inflammable and explosive characteristics.[2] It is also popular in many other industrial applications, such as metal anticorrosion and the synthesis of raw medicine.[3] Currently, kinds of common hydrazine derivatives have been found, such as isoniazid, isocarboxazide, hydralazine which is for hypertension and heart failure.[4] Despite its wide applications, hydrazine is extremely poisonous, and long-term exposure can cause fatal damage of the liver, kidney and central nervous system. Besides, it can be easily absorbed by oral, dermal or inhalation routes.[5] According to the U.S. Environmental Protection Agency (EPA), hydrazine has been classified as a probable human carcinogen, with an exposure limit of 10 ppb.[6,7]

So far, a variety of analytical techniques such as chromatography-mass spectrometric[8], titrimetric[9] and electrochemical methods[10] have been exploited for hydrazine. However, some of them were complex, time-consuming and/or needed special equipment.[11] Therefore, it is crucial to develop a new sensitive and reliable method for determination of hydrazine. In recent years, the design and development of fluorescent probes for the detection of important small molecules of biology and environment have attracted extensive attention owing to their high sensitivity and specificity. However, there were only a limited number of reports regarding fluorescent probes for hydrazine as of yet. For example, Swager *et al.* reported an amplifying fluorescent polymer which was reduced by hydrazine vapor, giving rise to an increase in fluorescence intensity.[12] Additionally, Tong *et al.* explored 5-chlorosalicylaldehyde as a special reactive probe for hydrazine through Schiff base reaction, yielding a strongly fluorescent product.[2] Chang *et al.* developed sequentially three fluorescence turn-on type probes for hydrazine by selective deprotection of levulinated coumarin,[13] dichlorofluorescein and resorufin acetates.[14] Fan[1] and Peng[3] *et al*. designed two reactive ratiometric fluorescent probes based on the special reaction of hydrazine with arylidenemalononitrile and cyanine dye derivative, respectively. Recently, Goswami *et al.* reported an excited state intramolecular proton transfer (ESIPT) probe based on 2-(2'-Hydroxyphenyl) benzothiazole (HBT) for the ratiometric detection of hydrazine in living cells.[15] However, the small-molecular probes mentioned above usually rendered several limitations such as commercially unavailable, difficult to be synthesized, pH dependent and working well only in organic co-solvent, as well as low sensitivity and poor specificity. To the

[a]*Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, China. Fax: +86 2583271269; Tel: +86 2593271269; Email: chenjl_4@hotmail.com; dibin@cpu.edu.cn; ddw888@vip.sina.com*

[b]*Key Laboratory of Drug Quality Control and Pharmacovigilance (China Pharmaceutical University), Ministry of Education, Nanjing 210009, China*

[1]*Jin-Long Chen and Dian-Yun Qu contributed equally to this work*

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best of our knowledge, no small organic probe for hydrazine working well in aqueous solution at neutral pH has been reported so far.

Herein, we explored 3, 6-diacetoxyfluoran (FDA), a readily commercially available compound for fluorescence "switch-on" detection of hydrazine in nearly pure water at pH 7.5 (5.0 mM, Tris buffer) with high sensitivity. Besides, this proposed method behaved excellent specificity toward hydrazine over other similar compounds containing amino-group. In addition, its excellent sensitivity and specificity ensured this method capable of analyzing hydrazine in four real samples and visualization of hydrazine vapor by naked-eye successfully.

Experiment section

Reagents and apparatus

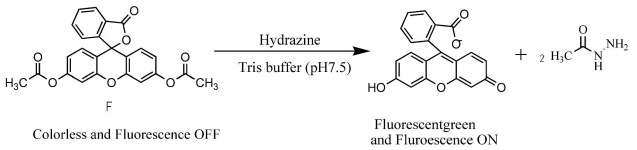
All of the chemicals used were at least analytical grade. Acetic anhydride, dehydrated alcohol, acetonitrile, triethanolamine, N, N-Dimethylformamide (DMF), triethanolamine, ammonia water were purchased from Nanjing Chemical Reagent Co., Ltd. Isoniazid, fluorescein and hydrazine were purchased from Aldrich Chemical Co., and hydralazine was purchased from D.K. Pharma Chem Pvt. Ltd(India). Plasma was provided by China Pharmaceutical University Hospital. Fluorescence spectra were obtained on a HITACHI 5JZ-0004 spectrofluorometer. Mass spectra were measured by Agilent 6224 TOF-LC/MS. IR spectrum was recorded on a JASCO FT/IR-4100 spectrofluorometer. And ^{1}H NMR spectrum was obtained on an AV300 NMR spectrometer. Distilled water obtained from a RO-DI water purification system (Pall Corporation) was used throughout this work.

Synthesis and character of FDA

According to previous literature,[16] 5 g fluorescein and 24 mL acetic anhydride were added to a 100 mL round flask, then the reaction mixture was heated to reflux and kept reacting under stirring for 2 hours. When temperature reduced to 70-80 ℃, the reaction mixture was poured into ice water under vigorous stir for prevention of clumps. The precipitate was filtered, and the crude product was recrystallized from dehydrated alcohol three times to give FDA as white powder (5.53 g, 86% yield) (Scheme S1). m. p. 205-206 ℃, FT-IR (KBr, cm^{-1}) 3035 ($\nu_{=C-H}$), 1761 ($\nu_{C=O}$), 1609 and 1496 ($\nu_{C=C}$), 1211 and 1160(ν_{C-O-C}) (Fig. S1). ^{1}H NMR (300 MHz, DMSO-d6) δ (ppm): 8.08 (d, J= 7.1 Hz, 1H), 7.85 (m, 2H), 7.43 (d, J= 7.3 Hz, 1H), 7.29 (s, 2H), 6.97 (m, 4H), 2.3 (s, 6H) (Fig. S2). ^{13}C NMR (300 MHz, DMSO-d6) δ (ppm): 168.75, 168.32, 152.10, 151.99, 150.77, 135.93, 130.49, 129.03, 125.42, 124.96, 124.07, 118.54, 116.12, 110.41, 80.97, 20.77(Fig. S3). HRMS (ESI): m/z calcd. for $C_{24}H_{16}O_7$ $[M+H]^+$: 417.0969, found: 417.0996; m/z calcd. for $C_{24}H_{16}O_7$ $[M+Na]^+$: 439.0788, found: 439.0789; m/z calcd. for $C_{20}H_{12}O_5$ $[M+H]^+$: 333.0763, found: 333.0770; m/z calcd. for $C_{20}H_{12}O_5$ $[M+Na]^+$: 355.0577, found: 355.0568.

Analytical procedure

To a 10 mL graduated test tube, 5 mL Tris buffer (10.0 mM, pH 7.5), a 200 μL hydrazine solution (2.5×10^{-2} mol L^{-1}) and 200 μL FDA solution (2.5×10^{-4} mol L^{-1}, dissolved with acetonitrile) were sequentially added. Finally, the graduated test tube was diluted to the volume with distilled water and mixed thoroughly immediately. Standing at room temperature for 20 minutes, the fluorescence spectra were measured by excitation/emission at 480 nm/515 nm. Blank solution was prepared similarly except hydrazine.



Scheme 1 Representative reaction mechanism of FDA with hydrazine

Detection of hydrazine in real samples

The proposed method was firstly used for determination of hydrazine in distilled water, tap water and pharmaceutical isoniazid samples. An aliquot of hydrazine was added to the above three solutions. Recoveries of hydrazine of three different concentrations were detected. We further tested recovery of hydrazine in plasma sample. An aliquot of 200 μL blank plasma and three different concentrations of hydrazine were added to centrifuge tubes, then, 600 μL acetonitrile was added sequentially, mixed thoroughly and centrifuged for precipitating proteins. The obtained supernatant was transferred to a 10 mL graduated test tube, 5 mL Tris buffer (10.0 mM, pH 7.5) and a 200 μL FDA solution were continually added to each test tube. Nextly the graduated test tubes were diluted to the volume with distilled water and mixed immediately. Standing at room temperature for 20 min, the fluorescence spectra were measured by excitation/emission at 480 nm/515 nm. Blank solution was prepared similarly except hydrazine. All measurements were performed in triplicate.

Results and Discussion

Optical characterization and reaction mechanism of FDA reaction with hydrazine

In the two decades, various reactive probes were extensively developed to sense small molecules by selective and usually irreversible chemical reactions coupled with signal transduction, induced by target analytes.[17] So far, few hydrazine-selective probes have been successfully constructed based on hydrazine-induced deprotection of fluorochromes. Hydrazinolysis of phenol acetates is a well-known process, thus we envisioned that hydrazine could readily remove the phenol acetate moiety of the fluorochrome to produce a deprotected product. As expected,

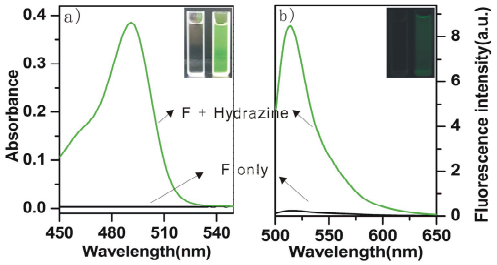


Fig.1 Typical UV-vis (a) and fluorescence spectra (b) of FDA in the presence and absence of hydrazine (Insets: photographs of FDA in the presence (right) and absence (left) of hydrazine under sunlight (a) and UV light of 365 nm (b), respectively. Experimental conditions: [F] = 5.0×10^{-6} M, [Hydrazine] = 5.0×10^{-4} M, Tris buffer solution (pH 7.5, 5.0 mM).

hydrazinolysis process of diacetoxyfluoran (FDA) was similar to a realization as in probes designed to indicate lipoidase activity and cellular vitality.[18] Herein, we exploited FDA to selectively detect gaseous and aqueous hydrazine by a fluorescence "switch-on" approach in an environment-friendly condition.

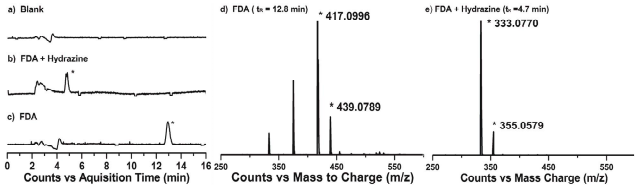


Fig.2 LC-MS spectra: total ion chromatogram (m/z: 200 to 800) for blank (a, 5.0 mM Tris buffer of pH 7.5 only), reaction mixture of 5.0×10^{-6} M FDA with 5.0×10^{-4} M hydrazine (b), only 5.0×10^{-6} M FDA (c), and corresponding MS spectra for only FDA (d), mixture of FDA reaction with hydrazine (e). LC-MS analyses were performed on an Agilent1260-TOF with a HPLC system coupled with TOF detector. The HPLC analysis was performed with a C18 analytical column (4.6 × 250 mm, 5 μm, Thermo) under 220 nm. The mobile phase was composited of water and acetonitrile (40/60, v/v) (flow rate, 1.0 mL min^{-1}). Scan mode: positive.

Free FDA was colorless and fluorescence off as well as very weak emission and absorption over 490 nm owing to the closed spirolactone form of fluorescein as shown in Scheme 1 and Fig.1 A prominent emission at 515 nm and simultaneous absorption at 490 nm resulting from the ring-opened fluorescein in aqueous solution were observed after the addition of hydrazine. Concomitantly, the fluorescence and color of the solution changed from dark to green as well as from colorless to green under UV illumination of 365 nm and Hydrazine was detected through ring-opening and hydrazinolysis of the phenol acetate moiety of FDA (Scheme 1). This process generated fluorescein, which exhibited its characteristic and fluorescent behaviours. The process of FDA transferring into fluorescein was directly identified by LC-MS. FDA free and its corresponding reaction product with hydrazine were analyzed by an Agilent 1260-TOF with a HPLC system coupled with TOF detector. Seen from Fig. 2, only a chromatographic peak with a retention time at 12.8 min for FDA was observed with m/z of 417.0996 (calcd. 417.0969) and 439.0789 (calcd.439.0788) which corresponded to $[M+H]^+$ and $[M+Na]^+$, respectively (Fig. 2c and d). A new chromatographic peak with a retention time at 4.7 min was observed concomitantly with a disappearance of peak at 12.8 min that the unique peak at m/z 333.0770 (calcd. 333.0763) corresponding to [Fluorescein + H]$^+$ when hydrazine was added to FDA solution.

Effect of pH on FDA and hydrazine system

In this work, effect of pH on FDA and hydrazine system was investigated. It is known that diacetate group of FDA molecule is highly pH-sensitive. So that, fluorescence of free FDA solution was recovered due to its hydrolysis reaction at high basic condition as shown in Fig.3. However, almost no emission was observed at pH lower than 7.0. A plot of fluorescence difference versus pH shown in Fig.3 exhibited that fluorescence enhancement significantly increased firstly and then slightly decreased. The maximum and stable fluorescence enhancement occurred in the range of pH 7.5 and 8.0. While an obvious

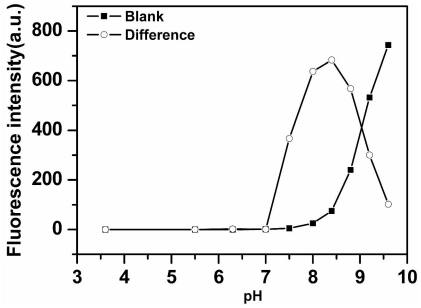


Fig.3 pH-dependent fluorescence intensity of free FDA and its response to hydrazine. Experimental conditions: [F] = 5.0×10^{-6} M, [Hydrazine] = 5.0×10^{-4} M.

enhancement of fluorescence blank of FDA was observed at pH higher than 8.0 due to hydrolysis reaction. In consideration of higher sensitivity and lower fluorescence blank, a Tris buffer (pH 7.5, 5.0 mM) was recommended throughout our work.

Kinetics property of reaction between FDA and hydrazine

As indicated in Fig.S4, upon the addition of 200 μL hydrazine (2.5×10^{-2} M) and 200 μL FDA solution (2.5×10^{-4} M) in a Tris buffer (pH 7.5, 5.0 mM), the FDA quickly transferred into strong fluorescent fluorescein as a product triggered by hydrazilysis approach. Fluorescent intensity of the reaction was investigated during 48 h, as shown in Fig.S4. A nearly linear temporal fluorescent enhancement was observed during the first 6 h, and the reaction tended to the balance after 12 h. Therefore, it is time-consuming to choose the reaction end point as detection time. Considering time-saving, we attempted to choose a much shorter time. Pre-experiment proved that satisfying precision, linearity and recovery can be achieved at 20th min. Under comprehensive consideration, we choose 20 min as detection time.

Analytical performances of the developed FDA-based probe for detection of hydrazine and analysis of four real samples

Linearity relationship of the proposed method

The emission profiles of 5.0 μM FDA and its reactive mixture of a series of hydrazine were collected at room temperature after standing for 20 min shown in Fig. 4. The fluorescence intensity at 515 nm enhanced gradually with the increase of hydrazine. A linear relationship was observed between fluorescence

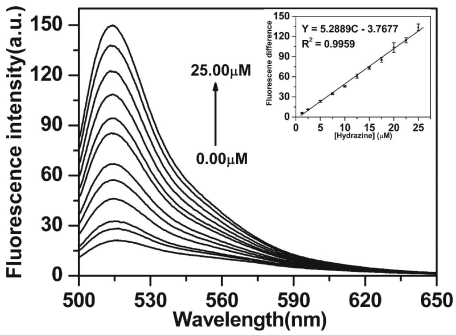


Fig.4 Fluorescence spectra of 5 μM FDA solution under excitation at 480 nm in the presence of various hydrazine concentrations in a pH 7.5 of 5.0 mM Tris buffer (0.00, 1.25, 2.50, 5.00, 7.50, 10.00, 12.50, 15.00, 17.50, 20.00, 22.50 and 25.00 μM. Inset: plot of the enhanced fluorescence intensity at 515 nm (ΔI) versus hydrazine concentration ([Hydrazine]).

enhancement and [Hydrazine] in the range of 1.25 to 25.00 μM without obvious change for emission profile. Thus, the detection limit (3 σ /slope) was calculated to be 31 nM, which is essentially lower than hydrazine limit provided according to EPA. The relative standard deviation of twelve replicate measurement samples was 5.4% for 12.5 μM hydrazine.

For comparative purpose, working conditions as well as the linear range and detection limit of several familiar fluorimetric methods for hydrazine detection were summarized in Table S1. It can be found clearly that five organic probes only worked well under the assistant of some amounts of organic solvent coupled with either high acidic or basic conditions. Furthermore, it was difficult to synthesize 2-(4-((4-(Benzo[d]thiazol-2-yl)phenyl)ethynyl)benzylidene)-malononitrile, Cy7A and Levulinated coumarin.

Specificity of FDA to hydrazine

We investigated the specificity of FDA toward hydrazine. As shown in Fig. S5 and S6, other cations represented by Na^+, K^+, Mg^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+} as well as anions such as Cl^-, SO_4^{2-}, NO_3^-, F^-, Br^-, I^- which were environmental and/or biological abundant, caused almost no changes to emission spectra of FDA. Furthermore, competition experiments on the fluorescence of the

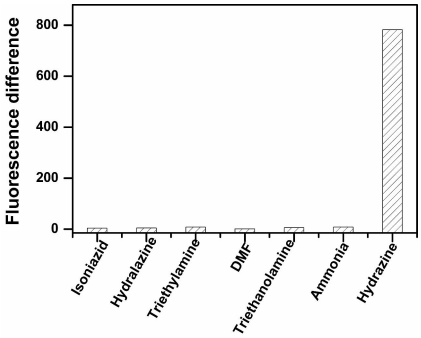


Fig.5 Fluorescence response of FDA to hydrazine and structurally related analogs. Experimental conditions: [F] = 5.0×10^{-6} M, [The analytes] = 5.0×10^{-4} M, Tris buffer (pH 7.5, 5.0 mM).

FDA-hydrazine system revealed hydrazine-induced fluorescence changes of FDA were not significantly altered in the presence of an equiv of coexisting common anions and metal cations except for Cu^{2+} and Fe^{3+} which might be due to the interaction of two metal ions with hydrazine in Fig. S6.[14] The interference from Cu^{2+} and Fe^{3+} could be effectively circumvented by using a chelating resin Chelex-100.[13] It was needed to note that other analogs structurally related to hydrazine such as isoniazid, hydralazine, triethylamine, triethanolamine, ammonia were also examined in our work (Fig. 5). Neglectable changes in the emission spectra of FDA under experimental conditions were found. Thus, all the above results established that FDA could detect hydrazine both qualitatively and quantitatively. Blank solution was prepared similarly except hydrazine. All measurements were performed in triplicate.

Analytical results of four real samples

The presented method was used for determination of hydrazine in distilled water, tap water, distilled water with 5.0×10^{-4} M isoniazid and plasma. An aliquot of hydrazine was added to the above four samples. Recoveries of hydrazine of the three water samples and plasma sample were summarized in Table 1. The detection of hydrazine in the above three solutions agreed well at hydrazine concentrations up to 20.00 μM. From Table 1, it can be seen that the results of recovery for four samples were satisfied, suggesting that the developed method was reliable and practical.

Table 1 Analytical results for detection of hydrazine in water, plasma and pharmaceutical samples

Samples	Hydrazine spiked (μM)	Found (mean ±σ, n=3, μM)	Recovery (%)
Distilled water	5.00	5.16±0.011	103.2
	12.50	13.55±0.019	108.4
	20.00	21.33±0.027	106.6
Tap water	5.00	5.71±0.028	114.1
	12.50	14.60±0.03	116.8
	20.00	23.70±0.034	118.5
Isoniazid[a]	5.00	4.75±0.033	96.6
	12.50	11.85±0.031	94.8
	20.00	19.09±0.005	95.5
Plasma sample	7.50	7.28±0.052	97.0
	12.50	11.48±0.007	91.8
	17.50	17.78±0.005	101.6

[a] The sample contained 0.5 mM isoniazid .

Paper -based visual detection of gaseous hydrazine by naked-eye

To develop a simple, cheap, portable paper-based sensor is an important goal for the instant on-site detection in practical applications. For a proof-of-concept demonstration, a piece of Nylon 66 microporous membrane with a pore size of 0.22 μm, cheap and easily available, was chosen as a substance for membrane-based visual detection of gaseous hydrazine by naked-eye that was firstly immersed in acetonitrile solution containing 25.0 mM FDA and dried for two minutes at room temperature. Then, the organic membrane coated with FDA molecules was tightly covered on the vial containing gaseous hydrazine with different concentrations ranging from 0.1% to 1.0 % for 10 min at room temperature. As shown in Fig. 6, distinct changes from colorless and blue into green quickly for both color and fluorescence obtained under sunlight and UV light of 365 nm could be readily distinguished with the naked eye.

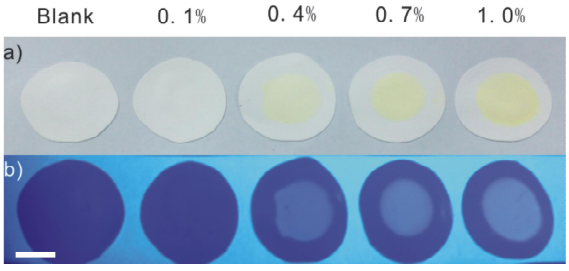


Fig. 6 Colorimetric and fluorescent visualization of FDA-coated organic membrane after exposure to different concentrations of hydrazine (0.1%, 0.4%, 0.7% and 1.0%) by naked-eye under the sunlight (a) and UV light of 365 nm (b), respectively. The scale bar is 1.0 cm.

Conclusions

In conclusion, 3, 6-diacetoxyfluoran (FDA), a commercially available reagent has been evaluated for "switch-on" fluorescent detection of hydrazine based on well-established hydrainolysis

transformed into fluorescein as a product with a distinctive signal alteration. It is noteworthy that the presented method was endowed with a wide linear response range, specificity for hydrazine and excellent sensitivity with a detection limit as low as 31 nM, as well as environment-friendly conditions. Furthermore, when coated on organic membrane, FDA could be used for sensitive detection of vaporized hydrazine with direct readout from the easy-to-visualize fluorescent and color changes. This proposed fluorescent probe permits reliable detection of trace hydrazine in four real samples containing distilled water, tap water, isoniazid, plasma samples.

Acknowledgment

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