Paper Strip-based Fluorometric Determination of Cyanide with an Internal Reference

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The rapid, selective, and sensitive determination of cyanide anion (CN^-) using a simple paper strip is highly attractive because cyanide is acutely lethal to living organisms via all routes of administration, including alcohol consumption and inhaling cigarette smoke. Here, a synthetic probe (**1**) was designed for the selective determination of cyanide. The probe displays rapid and large blue spectral change ($\Delta\lambda_{abs}$ =148 nm, $\Delta\lambda_{em}$ = 165 nm) with respect to target recognition. Probe **1** exhibits a strong push–pull electronic effect and comprises a dimethylaminoaryl group as a donor and malononitrile as an acceptor; the π-conjugation system can be destroyed by the Michael-type addition of cyanide at the electrophilic β-positions of the nitrile groups, resulting in the marked emergence of a peak at λ_{em} = 515 nm. The developed probe was successfully applied to a paper test strip because of its noticeable optical changes upon reaction with cyanide. The fabricated dumbbell-shaped paper strip with an internal reference allowed the cyanide detection, which is indispensable for quantitative analysis in point-of-care testing. The paper strip test showed selective response to cyanide, with a linear correlation in the range of 0–25 mM in a simple and cost-effective manner.

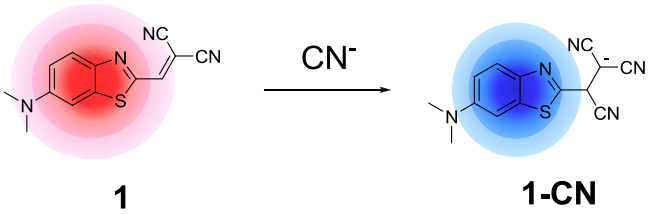
Keywords: Cyanide, Fluorescent probe, Paper strip, Point-of-care testing, Internal reference

Introduction

Cyanide is extremely toxic to mammals, primarily because it impairs the mitochondrial respiratory chain. This results from the strong coordination of cyanide to iron at the heme active site in cytochrome oxidase, whereby it inhibits the aerobic production of adenine triphosphate.[1,2] Cyanide poisoning is also closely related to cardiovascular and central nervous system disorders.[3] According to previous reports, the LD_{50} values of hydrogen cyanide for human intake and dermal exposure are 1.0 and 100 mg/kg, respectively.[4] Cyanide can cause serious problems when it is accidentally released in operations such as gold cyanidation and industrial organic chemistry for nylon production and in certain areas of architecture; therefore, cyanides should be controlled with extreme precaution.[5]

Various potentiometric,[6] voltammetric,[7] chromatographic,[8] or fluorescence detection methods[9–11] have been reported for cyanide determination. Although these techniques meet most legislative requirements, they are operationally complex and time-consuming and often require significant preconcentration and/or organic solvents. Consequently, alternative and more sensitive methods that can directly measure cyanide from sub-μg/L to mg/L levels in different matrices are desirable. Among them, fluorescence determination shows promise because of its favorable sensitivity, operating cost, and operating process. Jang *et al*. recently described a cyanide probe that utilizes the nucleophilic attack of cyanide, resulting in strong fluorescence in aqueous conditions.[9] Moreover, tremendous efforts have been devoted to the fluorescence determination of cyanides.[12] For several decades, paper-based sensors have attracted considerable attention because they can be easily utilized by the public for field testing.[13–15] Paper test strips are popular in the diagnostics field because of their low cost, light weight, portability, and ability to produce a rapid and easy-to-read answer. A simple point-of-care test for the rapid quantification of cyanide is also of great value in clinical examinations and forensic investigations because cyanide exposure can occur frequently in daily life.

Here, we report the quantitative determination of cyanide using a paper test strip that employs a cyanide-sensitive optical probe. The synthetic probe **1** has an electron push–pull-type structure containing a dimethylamino group as an electron donor and malononitrile as an electron acceptor. Probe **1** acts as a fluorescent chemodosimeter toward cyanide; the strongly nucleophilic cyanide reacts with the electrophilic methylenemalononitrile group of probe **1** to form **1**-CN via a Michael-type conjugate addition, thereby generating ratiometric optical signal changes (Scheme 1). The addition of the cyanide anion breaks the π-conjugation, which decreases the dipole moment and results in the large spectral blue shift in the probe **1**. These photophysical changes enable the naked-eye determination of cyanide;



Scheme 1. Cyanide probing concept of **1**.

thus, probe **1** can be utilized for detecting cyanide in paper-based sensors. We fabricated a dumbbell-shaped, simple paper device that allows the two-point referencing detection of cyanide. The rapid, quantitative determination of cyanide in the linear range of 0–25 mM was achieved on the internally referenced test strip in a simple and cost-effective manner.

Experimental

Synthesis Procedure for ((6-(dimethylamino)-1,3-Benzothiazol-2-yl)methylene)malononitrile (1). Probe **1** was prepared following a previously reported synthesis procedure (Scheme S1, Supporting Information).[16] To a solution of 6-(dimethylamino)-1,3-benzothiazole-2-carbaldehyde (**2**)[16] (160 mg, 0.78 mmol) in 2-propanol (9.75 mL), malononitrile (77.3 mg, 1.17 mmol), and pyridine (0.16 mL) were added. The mixture was stirred for 1.5 h at 90°C. The reaction mixture was cooled to room temperature (RT), and H_2O was subsequently poured into the mixture to quench the reaction. The mixture was extracted three times with $CHCl_3$, and the combined organic layer was dried over Na_2SO_4. All volatiles were evaporated under reduced pressure. The resulting crude product was purified by flash column chromatography with dichloromethane:methanol (20:1) as an eluent to give a violet solid (89% yield); ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.61 (s, 1H), 7.97 (d, J = 9.2 Hz, 1H), 7.37 (d, J = 2.4 Hz, 1H), 7.2 (dd, J = 9.2, 2.4 Hz, 1H), 3.07 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 151.2, 151.1, 151.0, 145.3, 141.4, 125.9, 116.4, 114.8, 113.9, 101.7, 80.4; HRMS (FAB$^+$, m-NBA): *m/z* calcd for $C_{13}H_{11}N_4S$: 255.0704, observed: 255.0705.

Photophysical Property Measurements. A 10-mM stock solution of **1** was prepared in DMSO; however, the solution appeared to be unstable even on exposure to ambient light because of the labile nature of the vinyl linker.[17] Thus, all the experiments were performed with the solution carefully wrapped in aluminum foil to protect the solution from ambient light. The solution was diluted to 5 μM in DMSO just before photophysical measurements. The cyanide titration experiment was performed by adding increasing amounts of tetrabutylammonium cyanide (TBAC, 0–25 μM) to the solution of **1** (5 μM in DMSO). Each mixture was measured using Beckman DU 800 spectrophotometer (Beckman Coulter, Ramsey, Minnesota, USA) and FP-6500 Spectrofluorometer (JASCO International Co. Ltd., Tokyo, Japan). Photophysical response to each analyte was estimated by adding each analyte (sodium salt, 2 equiv of cyanide; 20 equiv. of other analytes) into each aliquot of the solution of **1** (5 μM in DMSO). A competing experiment was performed by adding 2 equiv of cyanide into the mixture of all analytes (each 20 equiv) in **1** (5 μM in DMSO). The fluorescence experiment was performed by excitation at 400 nm (slit width: 5 × 5 nm).

Paper-based Sensor. A dumbbell-shaped hydrophilic pattern was tailored using Microsoft PowerPoint software (Figure S3). The cross-linking channel on the paper was approximately 1.6 cm in length and 0.2 cm in width. A large circle with a diameter of 0.7 cm and a small circle with a diameter of 0.5 cm were drawn at the top and bottom of the cross-linking channel, respectively. The tailored dumbbell shape was printed with wax-based solid ink on Whatman chromatography paper using a Xerox 8570DN inkjet printer. After the paper was heated by placing it on a hot plate with the wax side up for 15 s at 120°C and then cooled to RT, the dumbbell-shaped hydrophilic pattern was completely separated from the wax printed hydrophobic site.[18] A solution of probe **1** in acetonitrile (1.2 μL, 5 mM) was dropped onto a detection site (large circle; (a) in Figure S3) and dried for 5 min. Then, 10 μL of various NaCN solutions in water (0–100 mM) was introduced onto the sample loading site (small circle; (c) in Figure S3). The sample droplet reached the detection site through the cross-linking channel, and then, the paper device was dried for 1 h. The fluorescence intensity changes on the paper were measured by irradiation at 365 nm (6 W) using a hand-held UV lamp (ENF-260C; Spectroline, Westbury, New York, USA). The paper strip was irradiated with UV light and photographed. The image was then imported to the Adobe Photoshop CS6 software to digitize the changes in fluorescence. The images were then transformed to gray scale, and the mean fluorescence intensity was determined from the image histogram. To compensate for measurement errors caused by various light sources, an internal standard was introduced by measuring the intensity on the sample loading circle because both circles (*i.e.*, the detection and sample loading sites) will be equally affected by the light source.[19,20] Thus, all data were internally referenced by measuring the ratio of intensity at the detection site to the intensity at the sample loading site. To test the selectivity of the paper strips, 30 mM of CN^-, SH^-, HPO_4^{2-}, OAc^-, N_3^-, F^-, Cl^-, and Br^- solutions (sodium salt) were prepared in 100 mM PBS buffer. Each analyte solution (10 μL) was loaded onto the sample loading site, and the results were determined using the aforementioned method.

Results and Discussion

Design Concept of Probe. Our probe was designed to have an electron push–pull conjugated structure consisting of electron donor and acceptor moieties. Malononitrile

and 6-(dimethylamino)-1,3-benzothiazole were connected through a methylene linkage comprising electron-deficient sites on the β-positions of the nitrile groups. Cyanide anion is strongly nucleophilic and is thus expected to attack the most electron-deficient site (Scheme 1). Thus, the synthetic probe **1** is fully π-conjugated from the dimethylamino group to the malononitrile group. Upon the administration of cyanide via Michael-type addition to probe **1**, the electrophilic vinyl site is attacked by the strongly nucleophilic cyanide, consequently destroying the conjugated structure and decreasing the magnitude of the molecular dipole moment from **1** to **1**-CN (Figure S4).[9] The decreased dipole moment would alter the HOMO–LUMO energy band gap (Figure 6), changing the photophysical properties.

Synthesis and Photophysical Properties of 1. Probe **1** was prepared according to the previously reported synthesis procedure, with a 28% overall yield (Scheme S1).[16] As expected, **1** showed a reddish color in the DMSO solution because of the inherently strong electronic push–pull effect. Upon adding increasing amounts of cyanide, the absorbance of **1** at approximately 530 nm decreased, whereas the absorbance at approximately 382 nm increased. Three pseudo-isosbestic points were observed at 293, 324, and 437 nm, indicating a 1:1 reaction stoichiometry (Figure 1(a)). The absorbance change is completed upon the addition of 1 equiv of cyanide (inset of Figure 1(a)). Similar to the large spectral shift (148 nm) observed in the absorbance spectra, the fluorescence spectra exhibited ratiometric changes with pseudo-isoemissive points at 617 nm upon excitation at 400 nm, as well as with saturation upon the addition of 1 equiv cyanide (Figure 1(b)). Cyanide showed selective response to probe **1** over other competitive analytes, including SH^-, HPO_4^{2-}, OAc^-, N_3^-, F^-, Cl^-, and Br^- (Figure 2(a)). The reddish solution of **1** dramatically changed to a transparent solution upon the addition of cyanide, whereas other analytes showed no significant color changes (Figure 2(b)). The color change was confirmed by the UV–Vis spectra, in which the absorption maximum at approximately 530 nm changed only in the presence of cyanide (Figure S1). Thus, the ratiometric probe **1** simultaneously records two emission peaks at 550 and 680 nm in the presence and absence of cyanide, allowing the accurate quantitative analysis of cyanide. The probe shows an approximately eight-fold greater ratiometric fluorescence response (550/680 nm) to cyanide compared to the other analytes (Figure 2(a)). The fluorescence measurements in

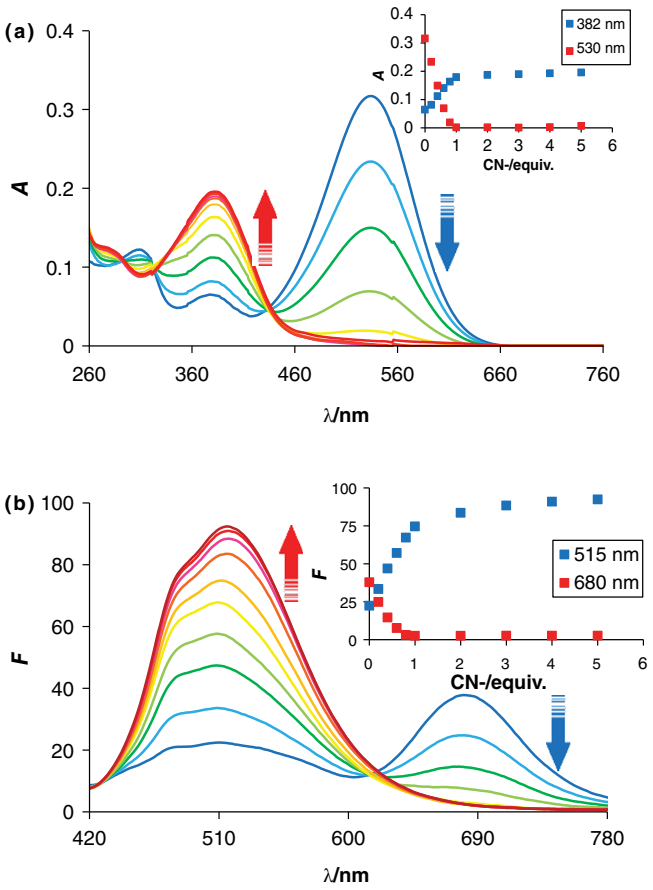


Figure 1. Absorbance (a) and fluorescence (b) changes of **1** (5 μM) upon titration with tetrabutylammonium cyanide (0–25 μM) in DMSO. Inset shows the absorbance intensities at 382 and 530 nm (a) and emission intensities at 515 and 680 nm (b) of **1** with cumulative cyanide addition (excitation: 400 nm, slit width: 5 × 5 nm).

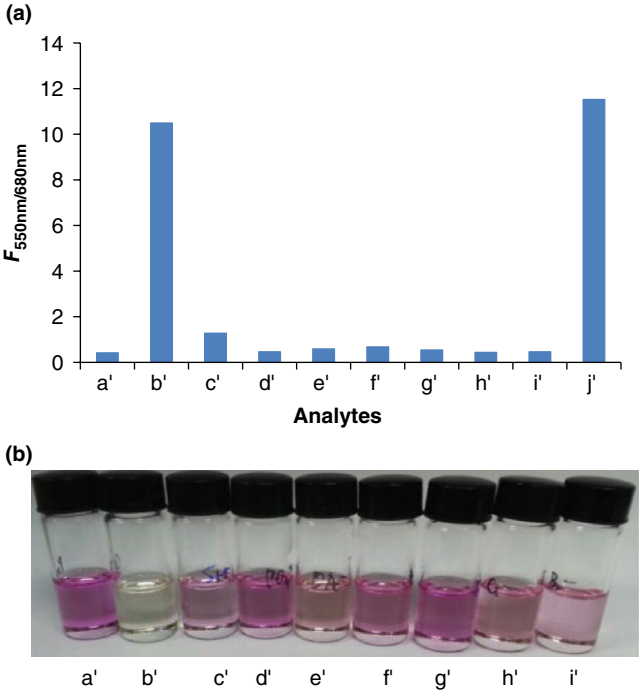


Figure 2. (a) Fluorescence intensity ratio ($F_{550\ nm}/F_{680\ nm}$) of **1** (5 μM) in the presence of analytes (20 equiv except for cyanide) in DMSO: (a′) probe **1**, (b′) CN^- (2 equiv.), (c′) SH^-, (d′) HPO_4^{2-}, (e′) OAc^-, (f′) N_3^-, (g′) F^-, (h′) Cl^-, (i′) Br^-, and (j′) all mixed analytes and the subsequent addition of 2 equiv cyanide (excitation: 400 nm, slit width: 5 × 5 nm). (b) The corresponding photographs.

the presence of the other competitive analytes revealed a selective response and reactivity for cyanide without any interference (Figures 2(a) and S2).

Paper Strip Sensor. As probe **1** showed a large spectral shift upon cyanide addition, it was expected to be applicable to a simple paper strip. We prepared a dumbbell-shaped lateral flow format on a filter paper, and three distributable sites (sample loading site, detection site, and cross-linking fluidic channel) were simply patterned on the paper using a wax-based inkjet printer. Aqueous samples introduced onto the sample loading site could travel to the detection site through the cross-linking fluidic channel, leading to a chemical reaction of the loaded sample with the imposed probe at the detection site (Figure S3). The fabricated paper device showed fluorogenic and colorimetric changes upon the addition of cyanide. Most importantly, the strips allowed a two-point referencing system by determining the ratio of the intensity at the detection site to the intensity at the sample loading site (a/c in Figure S3). This allows the compensation of possible errors caused by external light sources, which is indispensable for quantitative detection during field testing.[19,20] From the integrated fluorescence data processed by PC software, a linear correlation was obtained for cyanide concentrations in the range of 0–25 mM ($y = 0.0725x$, $R^2 = 0.99$; inset of Figure 3(a)). The limit of detection (LOD) was determined to be 1.59 mM (41.37 ppm; signal/noise ratio = 3, $n = 5$). Although the LOD did not reach a practically meaningful range (0.2 ppm in drinking water, US EPA), for the first time, the fabricated paper strip provides a model system to design matrices for *quantitative field tests for cyanide* with an internal reference. From comparative selectivity assays, the paper strip exhibits no significant response to other analytes, including SH^-, HPO_4^{2-}, OAc^-, N_3^-, F^-, Cl^-, and Br^-, indicating that the fabricated strip has the potential for cyanide determination in the field (Figure 4).

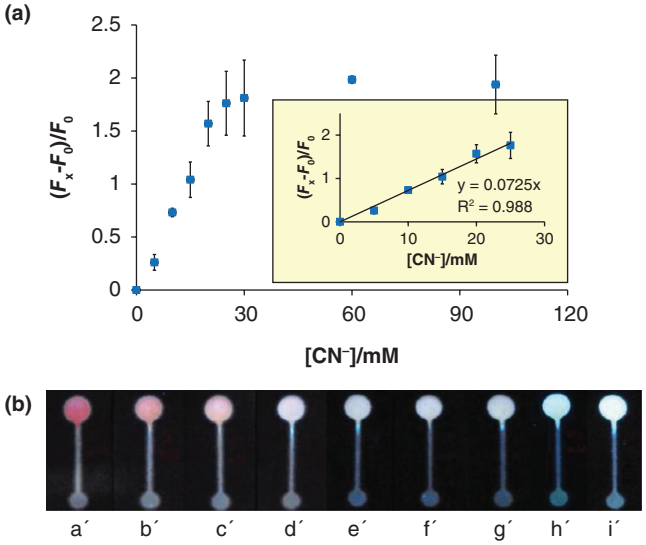


Figure 3. (a) Paper strip-based quantitative detection of cyanide (0–100 mM). The intensity was internally referenced by measuring the ratio of the intensities of the two circles (detection site/sample loading site). F_x = the intensity ratio of the two circles upon cyanide loading; F_0 = the intensity ratio on probe **1**. The plot shows the saturation point at 30 mM cyanide, and the error bar represents the standard deviation obtained from three sets of independent measurements. The inset yellow box shows a linear range from 0 to 25 mM of cyanide ($R^2 = 0.9891$). (b) The corresponding photographs according to the cyanide concentration under UV irradiation: (a′) 0, (b′) 5, (c′) 10, (d′) 15, (e′) 20, (f′) 25, (g′) 30, (h′) 60, and (i′) 100 mM of CN^-.

NMR Experiment. ^{1}H NMR was used to clarify the mechanism of cyanide detection. Upon the addition of increasing amounts of cyanide (0–1.0 equiv) into the solution of **1** in DMSO-d_6, one singlet peak at 8.6 ppm gradually disappeared, while a new singlet signal began to appear in the up-field region at 6.4 ppm. This result indicates that cyanide participates in the conjugate addition reaction at the most electron-deficient β-position of the nitrile groups to form **1**-CN, in which the β-olefinic carbon is converted to an sp^3 carbon attached to the incoming CN. Thus, the signal of the newly formed cyanomethine should appear far up-field compared to that of the original vinyl proton (Figure 5).

DFT Calculation. To obtain more insights into the mechanism, we examined the changes in the theoretical energy band gap using density function theory (DFT) computations at the B3LYP/6-31 + G* level of theory using the Gaussian09 program. The optimized geometry of **1** maintains a fully π-conjugated planar structure from the dimethylamino

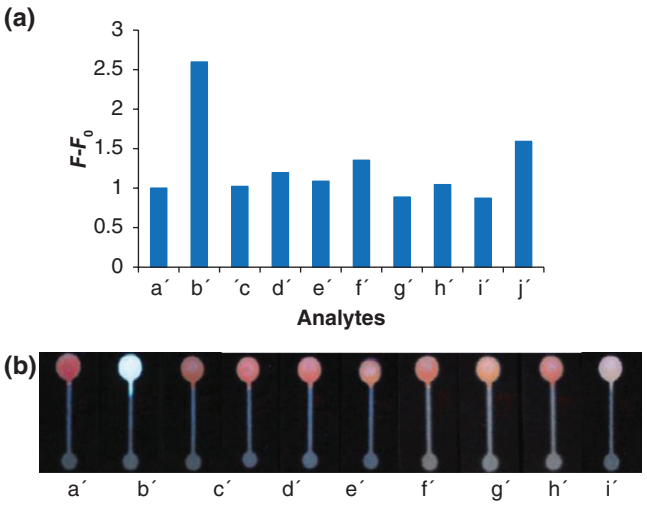


Figure 4. (a) Paper strip-based fluorogenic intensity ratio (F/F_0) upon administration of various anions (30 mM in 100 mM PBS buffer): (a′) probe **1**, (b′) CN^-, (c′) F^-, (d′) Cl^-, (e′) Br^-, (f′) HCO_3^- (g′) N_3^-, (h′) HPO_4^{2-}, (i′) OAc^-, and (j′) SH^-. The intensity was internally referenced by measuring the intensity ratio of the two circles (detection site/sample loading site). F = the intensity ratio of the two circles upon each analyte loading; F_0 = the intensity ratio on probe **1**. (b) The corresponding photographs.

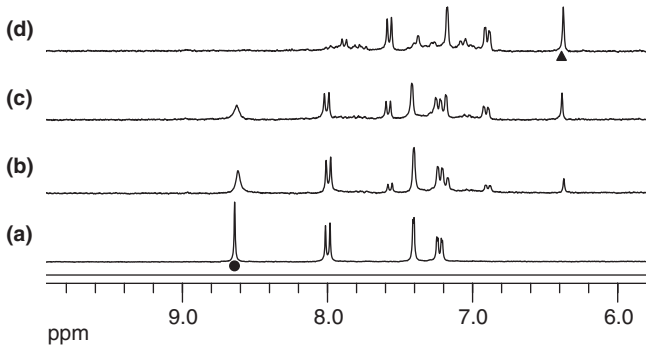


Figure 5. ^{1}H NMR spectra of **1** (10 mM) upon titration with tetrabutylammonium cyanide (TBAC) in DMSO-d_6: (a) 0, (b) 0.4, (c) 0.6, and (d) 1.0 equiv. of cyanide. The circle in (a) and triangle in (d) denote a vinyl proton in **1** and a cyanomethine proton in **1**-CN, respectively.

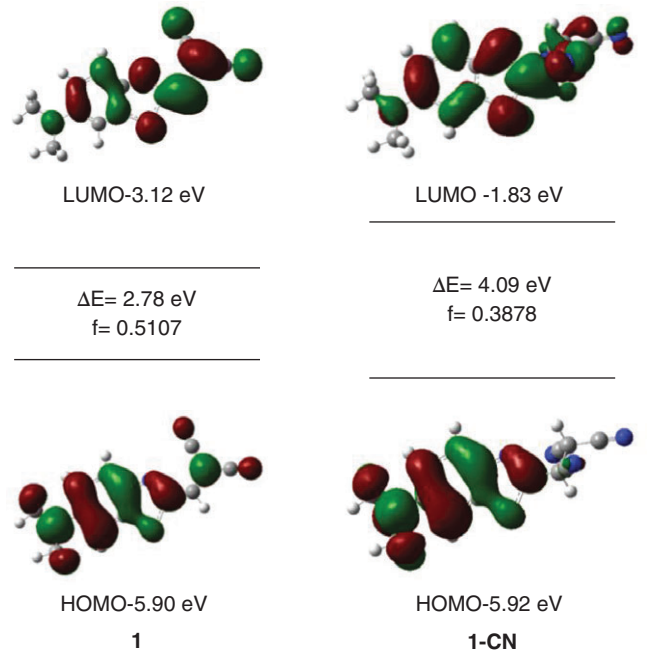


Figure 6. The HOMO and LUMO levels of **1** and **1**-CN and their corresponding frontier orbitals calculated using DFT. *f* is the calculated oscillator strength.

group to the methylenemalononitrile group, whereas the π-conjugation length in the cyanide adduct **1**-CN is shortened by the conversion of the π-conjugated methylene linker to the saturated aliphatic group, resulting in the breaking of the π-conjugated system (Figure S4). The DFT calculations reveal that the addition of cyanide changes the theoretical electronic transition energy (ΔE_{calcd}) from 2.78 eV in **1** to 4.09 eV in **1**-CN (Figure 6). The change in electronic transition energy ($\Delta\Delta E_{calcd}$ = 1.31 eV) can be converted to absorbance shift ($\Delta\lambda_{max,\ calcd}$ = 147 nm) via the Planck–Einstein relation[21]; this calculated shift is close to the experimentally observed change in absorbance, $\Delta\lambda_{max,\ exp}$ = 151 nm (Figure 1(a)).

Conclusions

In this study, we developed an electron push–pull-type chemodosimetric probe **1** that shows a selective optical response to cyanide. The large spectral shift of **1** was induced by alterations in the π-conjugated system, including the conjugation length and the magnitude of dipole moment, because of the nucleophilic attack by cyanide on the most electron-deficient sites, the β-positions of the nitrile groups in **1**. The large optical signal change of probe **1** upon the addition of cyanide was successfully developed into paper strips for determining cyanide in the field. The paper strip employing **1** showed selective sensing behavior toward cyanide. More importantly, the internally referenced system developed by the two-point measurement of the dumbbell-shaped strips facilitated the quantification analysis of cyanide in the field. The accurately described strip test would provide a reader-friendly guide about the point-of-care testing method for the determination of cyanide as well as other relevant targets.

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Supporting Information. Additional supporting information is available in the online version of this article.

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