A Novel Thiophene-Based Fluorescent Chemosensor for the Detection of Zn$^{2+}$ and CN$^-$: Imaging Applications in Live Cells and Zebrafish

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Abstract: A novel fluorescent turn-on chemosensor DHADC ((E)-3-((4-(diethylamino)-2-hydroxybenzylidene)amino)-2,3-dihydrothiophene-2-carboxamide) has been developed and used to detect Zn$^{2+}$ and CN$^-$. Compound DHADC displayed a notable fluorescence increase with Zn$^{2+}$. The limit of detection (2.55 ± 0.05 µM) for zinc ion was far below the standard (76 µM) of the WHO (World Health Organization). In particular, compound DHADC could be applied to determine Zn$^{2+}$ in real samples, and to image Zn$^{2+}$ in both HeLa cells and zebrafish. Additionally, DHADC could detect CN$^-$ through a fluorescence enhancement with little inhibition with the existence of other types of anions. The detection processes of compound DHADC for Zn$^{2+}$ and CN$^-$ were demonstrated with various analytical methods like Job plots, $^1$H NMR titrations, and ESI-Mass analyses.

Keywords: fluorescence chemosensor; zinc; cyanide; living cell; zebrafish

1. Introduction

The design of chemosensors with high selectivity and sensitivity has received great interest because they can recognize environmentally and biologically crucial metal ions and anions [1,2]. Among these ions, zinc ion is not only one of the essential metal ions in the human body, but is also the second richest transition metal ion [3,4]. It has large roles at catalytic sites of myriad Zn$^{2+}$-containing metalloenzymes and in DNA-binding proteins [5,6]. Meanwhile, an uncontrolled zinc concentration in the body creates a wide variety of troubles like epilepsy, Parkinson disease, and ischemic stroke [7]. Hence, it is of great significance to design chemosensors for the selective sensing of Zn$^{2+}$ in biological systems [8–11].

Recently, anions have attracted notable interest, owing to their various roles in clinical, environmental, and biological applications [12–14]. In particular, cyanide has been extensively used in numerous territories like synthetic fiber, gold mining, resin industries, and metallurgy [15–18]. Thus, the voluminous usage of cyanide is ineludible, and numerous industries yield about 140 k tons/year of cyanide [19–21]. However, cyanide acts as a strong poison. Its toxicity induces the susceptibility of binding to iron ion in metalloprotein cytochrome oxidase, blocking the electron transfer chain in mitochondria [22–24]. Moreover, high levels of cyanide can cause convulsions,
vomiting, loss of consciousness, and ultimately death [25,26]. Thus, it is essential to develop an effective sensing tool to recognize the cyanide level in living organisms and environments [27,28].

Among various analytical methods, a fluorescent method has attracted much attention due to its high selectivity, simplicity, and bioimaging ability [29–32]. Until now, a few fluorescence chemosensors for detecting both Zn$^{2+}$ and CN$^{-}$ were developed, but they are still rare. In addition, zinc fluorescent chemosensors for bioimaging in living cells and zebrafish are very rare (Table S1) [33–39]. Therefore, the development of fluorescent chemosensors with high selectivity and bioimaging ability in both living cells and zebrafish is needed.

Thiophene derivatives have been extensively utilized as a fluorescence signaling promoter to anions, organic acids, and metal ions [40,41]. Moreover, 4-diethylaminosalicylaldehyde moiety is an outstanding fluorophore that has a water-soluble electron-donor property [42,43]. Thus, we combined the two functional groups to design a novel and practical fluorescent sensor, which is expected to sense a particular analyte through a unique fluorescent property with bioimaging ability in both living cells and zebrafish.

Here, we demonstrate a novel and stable fluorescent chemosensor DHADC, comprised of 3-aminothiophene-2-carboxamide as a fluorescence-signaling group and 4-diethylaminosalicylaldehyde as an electron-donating group (Scheme 1). Chemosensor DHADC detected both Zn$^{2+}$ and CN$^{-}$ by fluorescent turn-on. To interpret their detecting systems, diverse analytical investigations like ESI-mass analyses, $^1$H NMR titrations, and Job plots were carried out.

Scheme 1. Synthesis procedure of DHADC.

2. Experimental

2.1. Reagents and Equipments

Chemicals were purchased from Sigma–Aldrich. A Varian spectrometer was used to obtain $^{13}$C (100 MHz) and $^1$H NMR (400 MHz) spectra. Fluorescence emission and UV-visible absorption spectra were recorded with Perkin Elmer spectrometers. ESI-MS data were obtained by a Thermo quadrupole ion trap. Fluorescence imaging in zebrafish and cells was obtained by a using fluorescence microscope (MDG36, Leica and EVOS FL, Thermo Fisher Scientific). * Caution for the use of cyanide: Skin, respiratory, and eye protection is required.

2.2. Synthesis of Sensor DHADC

$\text{[(E)-3-(((4-(diethylamino)-2-hydroxybenzylidene)amino)-2,3-dihydrothiophene-2-carboxamide)}$

$\text{3-Aminothiophene-2-carboxamide (1.1 mmol, 160 mg) and 4-diethylaminosalicylaldehyde (1 mmol, 200 mg) was dissolved in 12.0 mL of ethanol and blended for 8 h at 20 °C. The deep orange powder was given by filtration and purified with ether. The yield: 72% (230 mg);}$ $^1$H NMR: 11.42 (s, 1H), 8.77 (s, 1H), 8.01 (s, 1H), 7.72 (d, $J = 5.2$ Hz, 1H), 7.62 (s, 1H), 7.50 (d, $J = 8.8$ Hz, 1H), 7.38 (d, $J = 5.2$ Hz, 1H), 6.36 (d, $J = 8.8$ Hz, 1H), 6.13 (s, 1H), 3.38 (m, 4H), 1.13 (t, $J = 5.6$ Hz, 6H); and $^{13}$C NMR: 163.74, 162.29, 159.94, 159.93, 152.67, 149.72, 132.52, 129.91, 120.31, 109.47, 105.12, 97.26, 44.50, and 12.99. ESI-MS for [DHADC (C$_{16}$H$_{19}$N$_2$O$_2$S) + H$^+$]$: \text{calcd, 318.13 (m/z); found, 318.21 (m/z).}$
2.3. Fluorescence Titrations

1.0 mL of DMSO solvent was used to prepare a stock DHADC solution (1 × 10⁻² mmol, 3.2 mg) and 3.0 µL of the stock solution was diluted to 2.997 mL bis-tris buffer (1 × 10⁻² M, pH 7.0). 3–45 µL of zinc ion stock solution (0.1 M) dissolved in bis-tris buffer were added to DHADC (3.0 mL, 1 × 10⁻² mM). After blending them for 20 sec, fluorescence spectra were obtained. Both DHADC and DHADC-Zn²⁺ showed no decomposition for 7 h in buffer condition.

For CN⁻, 1.0 mL of DMSO solvent was used to prepare a stock DHADC solution (1 × 10⁻² mmol, 3.2 mg) and its eventual concentration (1 × 10⁻² mM) was given by adding 3 µL of stock DHADC solution (1 × 10⁻² M) into 2.996 mL MeCN. MeCN (1 mL) was employed to dissolve TEACN (tetraethylammonium cyanide, 33.1 mg (0.2 mmol)). 1.5–18 µL of cyanide (200 mM) were added to the compound DHADC (3.0 mL, 1 × 10⁻² mM). After mixing them for 20 sec, fluorescence spectra were obtained. The titrations for Zn²⁺ and CN⁻ were conducted three times for the average.

2.4. UV-vis Titrations

3 µL of a stock DHADC solution (1 × 10⁻² M) was transferred in fluorescent cell containing 2.997 mL bis-tris buffer. 6–42 µL of a zinc ion stock solution (0.1 M) in bis-tris buffer were added to the compound DHADC (3.0 mL, 1 × 10⁻² mM). UV-vis spectra were obtained after blending them for 20 s.

For CN⁻, 3 µL of a stock DHADC solution (1 × 10⁻² M) was added into 2.997 mL MeCN. 1.5–15 µL of this CN⁻ (0.2 M) in MeCN were added to the compound DHADC (3.0 mL, 1 × 10⁻² mM). UV-vis spectra were obtained after blending them for 20 s.

2.5. Job Plots

350 µL of a stock DHADC solution (1 × 10⁻² M) in DMSO was diluted to 49.65 mL bis-tris buffer for producing 0.07 mM. 0.3–2.7 mL of the diluted compound DHADC was added to fluorescent cells, respectively. 35 µL of a Zn²⁺ ion stock (1 × 10⁻¹ M) solution in bis-tris buffer was diluted to 49.97 mL bis-tris buffer. 2.7–0.3 mL of the diluted zinc ion was added to each DHADC in fluorescent cells. The total volume of each fluorescent cell was 3.0 mL. Fluorescence spectra were obtained after blending them for 20 s.

For CN⁻, 0.6 mL of a stock DHADC (1 × 10⁻² M) solution in DMSO was diluted to 29.4 mL MeCN/bis-tris buffer (95:5; v/v) to produce 2 × 10⁻³ M. 0.3–2.7 mL of the diluted compound DHADC was added to fluorescence cells, respectively. 30 µL of a CN⁻ stock solution (0.2 M) in MeCN was diluted to 29.97 mL MeCN/bis-tris buffer solution (95:5). 2.7–0.3 mL of the diluted cyanide was added to each DHADC in fluorescent cells. The total volume of each fluorescent cell was 3.0 mL. UV-visible spectra were obtained after blending them for 20 s.

2.6. Competition Tests

1 × 10⁻² mmol of Al(NO₃)₃ or NaNO₃ or Fe(ClO₄)₂ or In(NO₃)₃ or KNO₃ or Ga(NO₃)₃ or M(NO₃)₂ (M = Ni, Pb, Ca, Mg, Cu, Mn, Co, and Cd), or Fe(NO₃)₃ or Cr(NO₃)₃ was separately dissolved in 1.0 mL of bis-tris buffer. 42 µL of each metal ion (1 × 10⁻¹ M) was added into 3.0 mL bis-tris buffer to produce 140 equiv. Following the addition of 42.0 µL of a zinc ion stock solution (0.1 M), 3.0 µL of a stock DHADC solution (1 × 10⁻² M) was added into the fluorescent cells containing each metal ion. Fluorescence spectra were obtained after blending them for 20 s.

For CN⁻, 0.2 mmol of NaNO₂, tetracylammonium salts of F⁻, I⁻ Cl⁻, and Br⁻, Na₂S and tetrabutylammonium salts of N₃⁻, H₂PO₄⁻, OAc⁻, SCN⁻, and BzO⁻ was separately dissolved in 1.0 mL of bis-tris buffer. 15 µL (2 × 10⁻¹ M) of each anion was put into 3.0 mL MeCN/bis-tris buffer (95:5) to afford 100 equiv. Following the addition of 15 µL of TEACN solution (200 mM), 2 µL (1 × 10⁻² M) of compound DHADC was added into the fluorescent cells containing each anion. Fluorescence spectra were obtained after blending them for 20 s.
2.7. $^1$H NMR Titrations

DMSO-$d_6$ (2.8 mL) was used to dissolve compound DHADC (6.4 mg and 0.02 mmol) and 700 µL of DHADC was transferred to three NMR tubes, respectively. 0, 0.5, and 1 equiv of Zn$^{2+}$ dissolved in 1.2 mL of DMSO-$d_6$ solvent were added to each compound DHADC. $^1$H NMR spectra were obtained after blending them for 20 s.

For CN$^-$, DMSO-$d_6$ (2.8 mL) was used to dissolve DHADC (6.4 mg, 0.02 mmol) and 700 µL of DHADC was transferred to four NMR tubes, respectively. 0, 0.5, 1, and 2 equiv of TEACN dissolved in 2.4 mL of DMSO-$d_6$ solvent were added to each compound DHADC. $^1$H NMR spectra were obtained after blending them for 20 s.

2.8. Quantum Yields

The quantum yields of DHADC, DHADC-Zn$^{2+}$ and DHADC-CN$^-$ were determined with fluorescein ($\Phi_F = 0.92$) in basic ethanol as a reference fluorophore. By using calibration curves of fluorescein and their absorption spectrum, the concentrations of fluorescein corresponding to each DHADC, DHADC-Zn$^{2+}$, and DHADC-CN$^-$ species were calculated and expressed as fluorescein-DHADC, fluorescein-DHADC-Zn$^{2+}$, and fluorescein-DHADC-CN$^-$. The quantum yields were calculated with the following equation [44].

$$\Phi_{F,S} = \Phi_{F,R} \frac{A_R A_S}{A_S A_R} \left(\frac{n_S}{n_R}\right)^2$$

$\Phi_F$ is quantum yield, $A$ is absorbance, $F$ is the area of fluorescence emission curve, $n$ is refractive index of the solvent, $S$ is test sample, and $R$ is a reference sample.

2.9. Quantification of Zn$^{2+}$ in Real Samples

For fluorescent analysis in real samples, drinking water and tap water were obtained from our laboratory. The fluorescent analysis was carried out by adding 3.0 µL (10$^{-2}$ M) of compound DHADC and 0.30 mL of a bis-tris buffer (10$^{-2}$ M) to a 2.697 mL real sample solution having Zn$^{2+}$. Solutions were thoroughly blended and remained at 20 °C for 5 min. Their fluorescence spectra were obtained.

2.10. Imaging in Live Cells and Zebrafish

In media containing 100 mg/mL streptomycin, the Eagle Medium, 10.0% fetal bovine serum, and 100 U/mL penicillin HeLa cells were kept. The cells grew in a humidified condition at 37.0 °C under 5% CO$_2$. They were then put onto a 12 well plate (SPL Life Sciences, Pocheon, Gyeonggi-do, Republic of Korea) at a density of 1 x 10$^4$ cells/0.1 mL, cells were seeded and then incubated at 37.0 °C for 20 h. For fluorescent imaging tests, cells were treated with compound DHADC (dissolved in DMSO, 3 x 10$^{-2}$ mM) for 10 min, followed by the incubation of Zn(NO$_3$)$_2$ (dissolved in water, 5.0 mM) for 10 min. A EVOS FL fluorescent microscope was employed for imaging [emission 510 (±21) nm; excitation 470 (±11) nm].

The wild types of zebrafish (AB line) were incubated at 29 °C on a 14 h light/12 h dark cycle in E2 media (1 x 10$^{-5}$ M MgSO$_4$, 1 x 10$^{-3}$ M CaCl$_2$, 1.5 x 10$^{-4}$ M KH$_2$PO$_4$, 1.5 x 10$^{-2}$ M NaCl, 5 x 10$^{-4}$ M Na$_2$HPO$_4$, 1 x 10$^{-4}$ M KCl, 0.5 mg/L MB (methylene blue), and 0.7 mM NaHCO$_3$ at pH 7.2). Six-day-old zebrafish were prepared for fluorescence bio-imaging in vivo. Zebrafish were fed with only 5 x 10$^{-3}$ mM of DHADC in E2 media having 0.05% DMSO at 29 °C for 10 min. After the zebrafish were rinsed with E2 media to get rid of the remaining DHADC, the zebrafish were fed with the solution and had a wide range of concentrations of Zn$^{2+}$ (20, 50, 100, and 200 µM) for 10 min at 29 °C. They were rinsed with E2 media again and then 0.01% ethyl-3-aminobenzoate methanesulfonate was added for the fixed orientation of zebrafish. A fluorescent microscope (MDG36, Leica) was employed to image all zebrafish ($\lambda_{ex} = 450$–490 nm, $\lambda_{em} = 500$–550 nm). By using Icy software, the mean fluorescence intensity was determined.
3. Results and Discussion

Probe DHADC was provided by the reaction of 4-diethylaminoaldehyde and 3-aminothiophene-2-carboxamide in ethanol (72% yield, Scheme 1), and affirmed by $^{13}$C and $^2$H NMR and ESI-mass instrument.

3.1. Fluorescence Investigation of DHADC to Metal Cations

The sensing selectivity of DHADC was examined in the presence of various cations (Ni$^{2+}$, Mn$^{2+}$, Ga$^{3+}$, Fe$^{3+}$, Na$^+$, In$^{3+}$, Zn$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, Pb$^{2+}$, Mg$^{2+}$, Cr$^{3+}$, Co$^{2+}$, K$^+$, Al$^{3+}$, and Fe$^{2+}$) in bis-tris buffer (Figure 1). By adding each metal ion (140 equiv), Zn$^{2+}$ only exhibited a striking fluorescence increase (ca. 4500%) ($\lambda_{\text{ex}} = 446$ nm, $\lambda_{\text{em}} = 508$ nm). Instead, other cations did not increase the fluorescence. These outcomes illustrated that compound DHADC showed high discrimination towards Zn$^{2+}$.

![Figure 1. Fluorescent changes of DHADC (1 × 10$^{-5}$ M) with various cations (Al$^{3+}$, Ga$^{3+}$, In$^{3+}$, Cd$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Fe$^{2+}$, Mg$^{2+}$, Cr$^{3+}$, Hg$^{2+}$, Ag$^+$, Co$^{2+}$, Ni$^{2+}$, Na$^+$, K$^+$, Mn$^{2+}$, and Pb$^{2+}$, 140 equiv) with zinc ion was also analyzed by UV-visible titration (Figure S2). By adding each metal ion (140 equiv), Zn$^{2+}$ only exhibited a striking fluorescence increase (ca. 4500%) ($\lambda_{\text{ex}} = 446$ nm, $\lambda_{\text{em}} = 508$ nm). Instead, other cations did not increase the fluorescence. These outcomes illustrated that compound DHADC showed high discrimination towards Zn$^{2+}$.](image)

To examine the interaction between DHADC and Zn$^{2+}$, fluorescent titration of compound DHADC to zinc ion was executed (Figure 2). The emission (508 nm) of compound DHADC steadily increased and indicated a maximum at 140 equiv. ($\lambda_{\text{ex}} = 446$ nm). Quantum yields ($\Phi$) of 0.0003 ($\pm$0.0001) and 0.0135 ($\pm$0.0004) were determined for DHADC and DHADC-Zn$^{2+}$ (Figure S1; $\lambda_{\text{ex}} = 464$ nm). Binding type of DHADC with zinc ion was also analyzed by UV-visible titration (Figure S2). By adding Zn$^{2+}$ to compound DHADC, the peaks of 320 and 470 nm increased continuously, and that of 430 nm decreased. There were two definite isosbestic points (380 and 447 nm), meaning that the binding of DHADC to Zn$^{2+}$ formed one product.

The binding process of DHADC and zinc ion was proposed to be a 1:1 interaction with the analysis of Job plot (Figure S3; $\lambda_{\text{ex}} = 446$ nm, $\lambda_{\text{em}} = 508$ nm) [45]. The 1:1 interaction of DHADC-Zn$^{2+}$ was affirmed by the ESI-mass search (Figure S4). The mass data displayed that the peak of 458.00 (m/z) was reminiscent of [DHADC(-H$^+$) + Zn$^{2+}$ + DMSO]$^+$ (calculated at 458.05). With fluorescent titration, the association constant ($K$) for DHADC with Zn$^{2+}$ was given as $1.6 \times 10^3$ ($\pm$31) M$^{-1}$ by the equation of Benesi–Hildebrand (Figure S5) [46]. The constant was in the range of those ($K = 1–10^{12}$) of previously announced probes for Zn$^{2+}$ [47–49]. The binding process of compound DHADC with Zn$^{2+}$ was further inspected by the titration of $^1$H NMR (Figure 3). With addition of Zn$^{2+}$ (1 equiv.), the imine proton of 8.76 ppm was moved to downfield. At the same time, the protons of the thiophene moiety and the benzene ring were also moved to downfield. These results demonstrated that the N atom in the imine component and the O atom in the amide component may bind to Zn$^{2+}$. No shift of the proton signals was monitored with the addition of more Zn$^{2+}$ ions, which was indicative of...
a 1:1 binding of DHADC-Zn\(^{2+}\) species (Scheme 2). On the basis of the previous studies [34,50], the fluorescence turn-on mechanism of DHADC for Zn\(^{2+}\) might have the CHEF effect (chelation-enhanced fluorescence). During complexation of DHADC and Zn\(^{2+}\), the non-radiative transitions such as rotation and vibration were inhibited and the radiative transition was enhanced.

Figure 2. Fluorescent changes of DHADC (1 × 10\(^{-5}\) M) with different amounts of Zn\(^{2+}\) (from 0 to 150 equiv.). \(\lambda_{ex} = 446\) nm; slit width = 10 nm. Inset: Fluorescence intensity at 508 nm vs. the amounts of Zn\(^{2+}\).

Figure 3. \(^1\)H NMR titration of DHADC upon addition of Zn\(^{2+}\) (0, 0.5, and 1 equiv.).

To test the practicable capability of compound DHADC as a Zn\(^{2+}\) detector, the competitive study was executed in a mixture of Zn\(^{2+}\) (140 equiv.) and various interfering ions (140 equiv.; Al\(^{3+}\), Pb\(^{2+}\), Ga\(^{3+}\), Fe\(^{3+}\), K\(^{+}\), In\(^{3+}\), Ni\(^{2+}\), Cd\(^{2+}\), Mg\(^{2+}\), Fe\(^{2+}\), Cr\(^{3+}\), Na\(^{+}\), Ca\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), and Cu\(^{2+}\) ) (Figure S6; \(\lambda_{ex} = 446\) nm, \(\lambda_{em} = 508\) nm). Cu\(^{2+}\) fully interfered, and Fe\(^{3+}\), Cr\(^{3+}\), Fe\(^{2+}\), Co\(^{2+}\), and In\(^{3+}\) quenched 89%, 83%, 67%, 65%, and 22% of the fluorescence obtained with zinc ion alone. Therefore, the paramagnetic metal ions might be avoided for the applicability of the sensor in biological matrices. For practicable
applications, the pH response of DHADC-Zn\(^{2+}\) was investigated at a wide variety of pH (2–12) (Figure S7; \(\lambda_{ex} = 446\) nm, \(\lambda_{em} = 508\) nm). DHADC-Zn\(^{2+}\) species exhibited a momentous fluorescence enhancement between pH 7 and 10. Therefore, Zn\(^{2+}\) could obviously be sensed by the fluorescent analysis with compound DHADC over the physiologically and environmentally important pH scope of 7.0–8.4 [51].

![Scheme 2. Proposed structure of DHADC-Zn\(^{2+}\).](image)

We established a calibration plot for the quantitative measurement of Zn\(^{2+}\) by compound DHADC (\(\lambda_{ex} = 446\) nm, \(\lambda_{em} = 508\) nm). Compound DHADC exhibited a satisfactory linearity between its intensity and the concentration of Zn\(^{2+}\), indicating that compound DHADC could be a possible choice for the quantitative measurement of Zn\(^{2+}\). With the use of 3 \(\sigma/\)slope [52], the detection limit was determined by 2.55 (±0.05) \(\mu\)M (Figure 4), which was much lower than the guideline (76 \(\mu\)M) recommended by the World Health Organization (WHO) [53,54]. To confirm the practicable ability of compound DHADC to Zn\(^{2+}\) in environmental samples, the samples of tap and drinking water were chosen (Table 1; \(\lambda_{ex} = 446\) nm, \(\lambda_{em} = 508\) nm). Acceptable recoveries and relative standard deviation (R.S.D.) values were obtained for the samples. Thus, compound DHADC can be operational for the measurement of Zn\(^{2+}\) in practical applications.

![Figure 4. Fluorescence intensities (at 508 nm) of DHADC as a function of Zn(II) concentration ([DHADC] = 10 \(\mu\)mol/L and [Zn(II)] = 10–80 \(\mu\)mol/L). Conditions: All samples were conducted in bis-tris buffer solution (10 mM bis-tris, pH 7.0). \(\lambda_{ex} = 446\) nm; and slit width = 10 nm.](image)

\(y = 0.8514x + 21.973\)
\(R^2 = 0.9958\)

\(D.L. = 2.55 \pm 0.05 \mu\)M

In order to assess the sensing feasibility for biological applications of DHADC, we conducted fluorescent imaging experiments for sensing Zn\(^{2+}\) in living cells (Figure 5). We first incubated the HeLa cells with DHADC (30 \(\mu\)M) for 20 min. Then, the fluorescent emission in cells was not discovered without Zn\(^{2+}\). In contrast, the cells cultured with Zn\(^{2+}\) showed significantly increased fluorescence intensity. To further demonstrate the ability of DHADC in living organisms, the experiment for fluorescence imaging was carried out with zebrafish (Figure 6). When the zebrafish was incubated with DHADC (5 \(\mu\)M, a), there was no fluorescence signal. However, with increasing concentrations
(20–200 μM, b–e) of Zn$^{2+}$, the fluorescence signal gradually increased. By using Icy software, the mean fluorescent emission of the images was analyzed (Figure S8). The limit of detection was analyzed to be 21.44 (±2.6) μM. Thus, compound DHADC may be applied to intracellular sensing of Zn$^{2+}$ in living organisms.

**Table 1. Determination of zinc ion concentration**.

| Sample          | Zn(II) Added (μmol/L) | Zn(II) Found (μmol/L) | Recovery (%) | R.S.D. (n = 3) (%) |
|-----------------|-----------------------|-----------------------|--------------|--------------------|
| Drinking Water  | 0                     | 0                     |              |                    |
|                 | 20                    | 19.1 ± 0.6            | 95.5 ± 1.0   | 3.96 ± 0.5         |
| Tap Water       | 0                     | 0                     |              |                    |
|                 | 20                    | 19.7 ± 1.0            | 98.5 ± 1.4   | 1.51 ± 1.1         |

$^1$ Conditions: [DHADC] = 10 μM in 10 mM bis-tris buffer (pH 7.0).

**Figure 5.** Fluorescent imaging of HeLa cells incubated with DHADC and Zn(II). Cells were incubated with DHADC and Zn(II) for 20 min. (a$_1$, a$_2$): DHADC only; (b$_1$, b$_2$): DHADC with zinc ion. Conditions: [DHADC] = 30 μM; [Zn(II)] = 5 mM; 37 °C; and 5% CO$_2$. $\lambda_{\text{ex}}$ = 470 ± 11 nm. $\lambda_{\text{em}}$ = 510 nm. Scale bar was 50 μm.

**Figure 6.** Fluorescence images of zebrafish (6-day-old) incubated with DHADC followed by addition of zinc ion. Top line: bright-field image; middle line: fluorescent image; and bottom line: overlay image. (a$_1$–a$_3$): DHADC only; (b$_1$–b$_3$): DHADC with 20 μM zinc ion; (c$_1$–c$_3$): DHADC with 50 μM zinc ion; (d$_1$–d$_3$): DHADC with 100 μM zinc ion; and (e$_1$–e$_3$): DHADC with 200 μM zinc ion. [DHADC] = 5 μM. $\lambda_{\text{ex}}$ = 450–490 nm. $\lambda_{\text{em}}$ = 500–550 nm. Scale bar: 1 mm.
3.2. Fluorescence Studies of Compound DHADC to CN$^-$

The fluorescence sensing capability of DHADC to a variety of anions in bis-tris buffer/acetonitrile solution (5:95) was examined (Figure 7; $\lambda_{ex} = 459$ nm, $\lambda_{em} = 528$ nm). The fluorescent spectra of DHADC with diverse types of anions ($I^-$, $S_2^2-$, $H_2PO_4^-$, $Cl^-$, $N_3^-$, $F^-$, $BzO^-$, $Br^-$, $OAc^-$, $SCN^-$, and $NO_2^-$) showed very weak intensities. In contrast, there was a significant enhancement of fluorescence at 528 nm by adding 100 equiv. of CN$^-$). These outcomes indicated that compound DHADC could have a potential function as a choosy fluorescence receptor for CN$^-$. 

![Figure 7](image_url)

**Figure 7.** Fluorescent changes of DHADC (1 $\times$ 10$^{-5}$ M) with other anions (OAc$^-$, $F^-$, $Cl^-$, $Br^-$, $I^-$, $H_2PO_4^-$, $BzO^-$, $N_3^-$, $SCN^-$, $NO_2^-$, and $S_2^2-$, 100 equiv.; $\lambda_{ex} = 459$ nm; slit width = 10 nm).

To examine the influence of increasing levels of CN$^-$ to DHADC solution, the fluorescence titration was carried out (Figure 8; $\lambda_{ex} = 459$ nm, $\lambda_{em} = 528$ nm). When the CN$^-$ (0–120 equiv.) was added into DHADC solution, the fluorescence emission continuously increased at 528 nm and showed a maximum with 100 equiv. Quantum yields ($\Phi$) of 0.0063 ($\pm$0.0004) and 0.1118 ($\pm$0.0003) were analyzed for DHADC and DHADC-CN$^-$ (Figure S1). The binding character of compound DHADC with CN$^-$ was inspected by UV-visible titration test (Figure S9). With the addition of cyanide to compound DHADC, the peaks at 315 and 475 nm increased consistently, and 390 nm decreased continuously with two definite isosbestic points (345 and 425 nm).

![Figure 8](image_url)

**Figure 8.** Fluorescent changes of DHADC (1 $\times$ 10$^{-5}$ M) with different amounts of cyanide (from 0 to 120 equiv.). $\lambda_{ex} = 459$ nm; slit width = 10 nm. Inset: Fluorescence intensity at 528 nm vs. the amounts of cyanide.
To investigate the binding mode of DHADC and CN\(^-\), Job plot analysis was performed (Figure S10; \(\lambda_{ex} = 459\) nm, \(\lambda_{em} = 528\) nm) \([45]\). This result showed a 1:1 complexation, which was affirmed by ESI-MS analysis (Figure S11). Addition of CN\(^-\) (1 equiv.) into compound DHADC exhibited the production of the [DHADC - H\(^+\)] \([m/z: 316.21; calculated at 316.11]\). With the results of the fluorescent titration, the \(K\) value for DHADC with CN\(^-\) was given as 1.6 \(\times\) 10\(^3\) \((\pm 50)\) M\(^{-1}\) (Figure S12). The detection limit (3\(\sigma\)/slope) was determined by 44.6 \((\pm 1.5)\) \(\mu\)M (Figure 9) \([52]\).

![Figure 9](image)

**Figure 9.** Determination of the detection limit of DHADC (10 \(\mu\)M) for CN\(^-\) based on the change of intensity at 528 nm. \(\lambda_{ex} = 459\) nm; slit width = 10 nm.

To elucidate the detection process of compound DHADC with CN\(^-\), we carried out the titration experiments of \(^1\)H NMR (Figure S13). The proton of the hydroxyl component did not show up because of the possible inter or intra-molecular hydrogen bonds \([55]\). With the addition of CN (2 equiv.) to compound DHADC, all protons of the thiophene group and the benzene ring shifted to upfield. In contrast, one of the amide protons (H\(_3\)) was shifted to downfield, suggesting that the H\(_3\) proton might hydrogen bond to CN\(^-\) or HCN species (Scheme 3). These outcomes implied that the negative charge generated from the deprotonation of compound DHADC by cyanide was delocalized through the DHADC \([56]\). No movement of the proton signals was detected with addition of more amounts of CN\(^-\) (>2 equiv.). On the basis of the previous studies and our experimental data \([34,57,58]\), we can propose that the deprotonation of DHADC could cause the suppression of ICT (intramolecular charge transfer), which induces fluorescence turn-on of DHADC-H\(^+\) species. With the analysis results of ESI-mass, \(^1\)H NMR study and Job plot, the possible recognizing process of compound DHADC with CN\(^-\) was depicted in Scheme 3.

![Scheme 3](image)

**Scheme 3.** Proposed sensing mechanism of DHADC for CN\(^-\).

To inspect the inhibition of different types of anions, the competitive tests were achieved and are shown in Figure 10 (\(\lambda_{ex} = 459\) nm, \(\lambda_{em} = 528\) nm). Compound DHADC was mixed with CN\(^-\) (100 equiv.) and a wide variety of anions (S\(^2\)-, F\(^-\), BzO\(^-\), Cl\(^-\), SCN\(^-\), Br\(^-\), NO\(_2\)-, OAc\(^-\), N\(_3\)-, H\(_2\)PO\(_4\)-, and I\(^-\); 100 equiv.). Some inhibition was observed with F\(^-\), but its fluorescence was still discernible.
These observations illustrated that compound DHADC may be an excellent selective fluorescence detector for CN\textsuperscript{−}.

![Figure 10](image)

**Figure 10.** Competitive test of DHADC (1 × 10\textsuperscript{−5} M) to cyanide (100 equiv.) with other anions (100 equiv.). \(\lambda_{ex} = 459\text{ nm};\) slit width = 10 nm.

4. Conclusions

We demonstrated a unique fluorescent turn-on probe DHADC having a thiophene moiety. Compound DHADC could selectively sense Zn\textsuperscript{2+} and CN\textsuperscript{−} through fluorescence enhancement. Binding ratios of compound DHADC with Zn\textsuperscript{2+} and CN\textsuperscript{−} were proposed to be 1:1, with the analysis of ESI-mass data and Job plots. Detection limits for zinc ion and CN\textsuperscript{−} were 2.55 (±0.05) \(\mu\text{M}\) and 44.6 (±1.5) \(\mu\text{M}\), respectively. The value for zinc ion was far below the standard (76 \(\mu\text{M}\)) of the WHO. Importantly, compound DHADC could be used to analyze zinc ion in water samples and to image zinc ion in both zebrafish and live cells. Additionally, compound DHADC could detect CN\textsuperscript{−} with little interference of competitive anions. Moreover, the detection processes of DHADC with Zn\textsuperscript{2+} and CN\textsuperscript{−} were proposed through \(^{1}\text{H}\) NMR titrations and ESI-Mass analyses. Therefore, the results observed in this study illustrate that DHADC can be a detector to selectively detect Zn\textsuperscript{2+} and CN\textsuperscript{−} by the fluorescent turn-on method in aqueous and living organisms.

**Supplementary Materials:** The following are available online at [http://www.mdpi.com/1424-8220/19/24/5458/s1](http://www.mdpi.com/1424-8220/19/24/5458/s1), Table S1: Examples of chemosensors for detecting both Zn\textsuperscript{2+} and CN\textsuperscript{−}, Figure S1: (a) Fluorescent (\(\lambda_{ex} = 464\text{ nm}\)) and (b) absorption spectra of DHADC (3 \(\mu\text{M}\)), DHADC+Zn\textsuperscript{2+} (140 equiv.) and fluorescein. (c) Fluorescent (\(\lambda_{ex} = 459\text{ nm}\)) and (d) absorption spectra of DHADC (10 \(\mu\text{M}\)), DHADC+CN\textsuperscript{−} (100 equiv.) and fluorescein; slit width = 10 nm, Figure S2: UV-vis absorption spectra of DHADC (10 \(\mu\text{M}\)) obtained during the titration with Zn(NO\textsubscript{3})\textsubscript{2} (0−140 equiv.), Figure S3: (a) Fluorescence spectra of Job plot for the binding of DHADC with Zn\textsuperscript{2+}. (b) Intensity at 508 nm was plotted as a function of the molar ratio [Zn\textsuperscript{2+}]/([DHADC] + [Zn\textsuperscript{2+}]). The total concentration of zinc ion with DHADC was 7.0 \(\times\) 10\textsuperscript{−5} M. \(\lambda_{ex} = 446\text{ nm};\) slit width = 10 nm, Figure S4: Positive-ion electrospray ionization mass spectrum of DHADC (100 \(\mu\text{M}\)) upon addition of 1 equiv. of Zn\textsuperscript{2+}, Figure S5: Benesi-Hildebrand equation plot (intensity at 508 nm) of DHADC, assuming 1:1 stoichiometry for association between DHADC and Zn\textsuperscript{2+}. \(\lambda_{ex} = 446\text{ nm};\) slit width = 10 nm, Figure S6: Competitive selectivity of DHADC (10 \(\mu\text{M}\)) toward Zn\textsuperscript{2+} (140 equiv.) in the presence of other metal ions (140 equiv.). \(\lambda_{ex} = 446\text{ nm};\) slit width = 10 nm, Figure S7: Fluorescence intensities (at 508 nm) of DHADC and DHADC-Zn\textsuperscript{2+} complex, respectively, at different pH values (2−12). \(\lambda_{ex} = 446\text{ nm};\) slit width = 10 nm, Figure S8: Quantification of mean fluorescence intensity in Fig. 6 (a\textsubscript{2}, b\textsubscript{2}, c\textsubscript{2}, d\textsubscript{2} and e\textsubscript{2}), Figure S9: UV-vis absorption spectra of DHADC (10 \(\mu\text{M}\)) obtained during the titration with CN\textsuperscript{−} (0−100 equiv.), Figure S10: Job plot for the binding of DHADC with CN\textsuperscript{−}. Absorbance at 440 nm was plotted as a function of the molar ratio [CN\textsuperscript{−}]/([DHADC] + [CN\textsuperscript{−}]). The total concentration of CN\textsuperscript{−} with DHADC was 2.0 \(\times\) 10\textsuperscript{−5} M, Figure S11: Negative-ion electrospray ionization mass spectrum of DHADC (100 \(\mu\text{M}\)) upon addition of 1 equiv. of CN\textsuperscript{−}, Figure S12: Benesi-Hildebrand equation plot (intensity at 528 nm) of DHADC, assuming 1:1 stoichiometry for association between DHADC and CN\textsuperscript{−}. \(\lambda_{ex} = 459\text{ nm};\) slit width = 10 nm, Figure S13: \(^{1}\text{H}\) NMR titration of DHADC with CN\textsuperscript{−}. 

44.6 (±1.5) \(\mu\text{M}\).
Author Contributions: M.S.K. and C.K. provided the initial idea for this work; D.Y., J.B.C., H.S., H.L. and M.K. contributed to the collection and analysis of field test data; K.-T.K. and M.H.L. contributed to the analyses of results; M.S.K. and C.K. wrote the paper.

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