A Novel Coumarin-based Fluorescent Probe with Aggregation Induced Emission for Detecting CN⁻ and its Applications in Bioimaging

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Abstract
Although cyanogen ion (CN⁻) plays important role in industry which also bring acute environmental pollution. More serious, trace CN⁻ enters the human body can cause serious consequences and even death. Therefore, it is of great significance to detect trace CN⁻ with high sensitivity. Herein, a novel aggregation-induced emission (AIE) probe C-BH was synthesized based on coumarin matrix. Probe C-BH showed high selectivity and sensitivity toward CN⁻ by dual channel response due to the excited state intramolecular proton transfer (ESIPT). The low detection limit was calculated to be 0.05 µM. Moreover, probe C-BH was successfully used for imaging CN⁻ in living cells and zebrafish due to its low toxicity and excellent optical properties.

Keywords Cyanogen ion · Aggregation-induced emission · Excited state intramolecular proton transfer · Imaging · Zebrafish

Introduction
Cyanogen ion (CN⁻) is notorious for its strong toxicity [1]. As we know, trace CN⁻ can cause serious damage to the central nervous system by combining with Fe³⁺ of heme unit in hemoglobin [2]. Generally speaking, the concentration of CN⁻ in blood should be less than 20 µM to ensure the safety of organism, excessive CN⁻ may trigger serious adverse physiological reactions and lead to death [3–5]. Therefore, accurate detection of CN⁻ concentration in living organisms is of great significance to maintain the vitality of living organisms. So far, different analytical such as high performance liquid chromatography, mass spectrometry, inductively coupled plasma atomic emission spectrometry, and atomic absorption spectroscopy were exploited for monitoring CN⁻ and made some progress [6–10]. However, some defects such as high expenses, time-consuming and complicated operations made these measurement not easy to promote. It is urgent for developing new detection methods to overcome these defects and realize simple, rapid and high sensitivity detection of trace CN⁻.

In recent decades, fluorescence analysis has proved to be an important detection method in the field of life science due to its advantages in simple operation, non-destructive and rapid detection [11–15]. Fluorescence analysis was applied to the determination of CN⁻ in living organisms can effectively solve the difficulties as above mentioned. So far, only a few CN⁻ fluorescent probes were reported and most of them were single channel detection which easy to be affected by environment and instrument background. As we know, one probe possess ESIPT features can not only provide large Stoke shift to avoid the influence of fluorescence self-absorption, but also offer dual channel detection which makes fluorescence detection more accurate and effective [16–20]. In view of the toxicity of trace CN⁻, construction of dual channel probe for detection of CN⁻ could provide a built-in correction and thus make the analysis more accurate.

Since the discovery of AIE phenomenon by Tang’s Group in 2001, a large number of AIE-gens fluorescent probes have been developed which expanded the application in biological system greatly [21–24]. However, AIE probes were rarely used in cyanide detection which were not conducive to the development of this field, so it is necessary to develop AIE probes for the detection of CN⁻. In addition, as a kind of
natural products, coumarin has excellent photoelectric and low toxicity which can be used in photoelectric materials, sensors and bioimaging field. Based on the above considerations, in this paper, coumarin was selected as fluorescent matrix through Schiff base reaction, a novel fluorescent probe C-BH with AIE properties was obtained which showed “switch-on” phenomenon to CN− with high selectivity, high sensitivity and low detection.

Experimental Section

Materials and Methods

Unless otherwise specified, all reagents are purchased from commercial suppliers and without further purification is required for use. 7-Hydroxy-4-methyl-2H-chromen-2-one, hexamethylenetramine, acetic acid, benzophenone hydrazone, hydrochloric acid and ether was obtained from Adamas-beta® (Shanghai, China). 1H NMR and 13C NMR experiments were performed with an AVANCE III HD AN-400 MHz spectrometer (Bruker, Germany). Mass spectra were recorded on a LCMS-2020 spectrometer (Shimadzu, Japan). UV–vis spectra were recorded on a Perkin Elmer Lambda 1050+ spectrometer (PerkinElmer, USA). Fluorescence spectra were measured on a PE LSS55 fluorescence spectrophotometer (PerkinElmer, USA). The purity of the synthetic compound for spectroscopic measurement was detected via HPLC (1525 instrument, Waters, America) equipped with a UV/Visible detector.

Synthesis of C-BH

The synthetic route for preparing probe C-BH is given in Scheme 1. The intermediate 1 was synthesized according to the previous report [25]. Probe C-BH was obtained by simple preparation. Briefly, a mixture of the intermediate 1 (0.1632 g, 0.8 mmol) and benzophenone hydrazone (0.1963 g, 1 mmol) was dissolved in ethanol (25 mL). A drop of acetic acid was added and the mixture was refluxed for 6 h. The orange solid was precipitated, cooled, filtered and washed with cold ethanol to obtain the product (0.25 g, yield: 81.8%, purity 99.7%). 1H NMR (400 MHz, DMSO-d6) δ 12.05 (s, 1H), 9.20 (s, 1H), 7.70 (t, J = 8.1 Hz, 3H), 7.62 – 7.44 (m, 6H), 7.32 (dd, J = 6.7, 3.0 Hz, 2H), 6.78 (d, J = 8.9 Hz, 1H), 6.24 (s, 1H), 2.36 (s, 3H). 13C NMR (101 MHz, DMSO-d6) δ 169.45, 162.63, 159.49, 158.18, 154.32, 153.84, 136.61, 135.54, 132.02, 130.53, 129.77, 129.26, 129.21, 129.17, 128.51, 113.72, 112.22, 111.31, 105.72, 18.80. ESI–MS m/z: [probe]− calcd for C24H20N2O3 381.13; Found 381.20. The Correlation spectrum were showed in Figs. S1–S4.

Optical Measurements

Stock solutions of probe C-BH (10 mM) was prepared in DMSO solution. In addition, Ag+, Co2+, Fe3+, Cr3+, Pb2+, Zn2+, Cys, CH3COO−, Cl−, NO2−, NO3−, H2PO4− were prepared in distilled water from their corresponding salts, respectively. The fluorescent and absorption changes were recorded at room temperature each time.

Cell Culture

The HeLa cells were grown at 37 °C in a humidified 5% CO2 incubator using DMEM (Dulbecco’s modified eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum) for 24 h. Four groups of cells were studied. One group cell was incubated with probe C-BH (10 μM) and used as control group, the other three groups were treated with different concentrations of CN− (5, 10 and 15 μM) after incubated with probe C-BH for another 30 min.

Fluorescence Imaging in Zebrafish

Zebrafish research was approved by the NNU animal ethics and welfare Commission and was conducted in accordance with the European guidelines for the care and use of laboratory animals. Zebrafish is provided by Shanghai FishBio Co., Ltd. 5-day-old zebrafish was incubated with 10 μM the probe C-BH for 0.5 h. After removing the excess probe with PBS, zebrafish were further treated with CN− (5, 10, 15 μM) for 30 min. The zebrafish were only treated with probe C-BH (10 μM) was used as the control group. The imaging was performed in the green channel (488 nm) and blue channel (405 nm).
Results and Discussion

Synthesis of Probe C-BH

As a kind of excellent fluorescent matrix, coumarin received much attention and have been used in sensor field widely [26–29]. In this paper, coumarin was selected as fluorescent parent, benzophenone hydrazone was introduced by Schiff base reaction for giving probe C-BH with high yields. The formed “C=N” not only endows probe C-BH with tetra-tyrene like structure and engendered AIE properties, it also can be used as the action site of CN− which realized the original intention of the design.

AIE Properties

With probe C-BH in hand, we first evaluated the AIE performance of the probe C-BH in DMSO and H2O mixtures with different water fractions. The result was showed in Fig. 1a, probe C-BH showed weak fluorescence when the proportion of H2O is less than 50%, with the increase of water content, the fluorescence intensity of probe C-BH synchronous enhancement. The fluorescence intensity reaches the maximum when the water content reached 85% and showed strong yellow fluorescence. Scale diagram of fluorescence peak intensity and ratio of water was exhibited in Fig. 1b. The molecular aggregation morphology was further characterized by scanning electron microscopy (SEM), probe C-BH was dissolved in DMSO and DMSO / H2O (=1:4 Vol) respectively. Dispersed spherical particles can be observed when the probe C-BH was dispersed in pure DMSO solution (Fig. S5a) while the solution was changed to DMSO/H2O, obvious accumulation of particles were appeared (Fig. S5b) which proved probe C-BH has AIE feature. The average particle size of dispersed spherical particles was about 175 nm which was carried by DLS and showed in the Fig. S6.

Spectral Properties

UV–vis absorption spectrum of probe C-BH was further researched, in the absent of CN−, probe C-BH showed a single absorption peak at 325 nm, while 60 μM CN− was added, the peak located at 325 nm was disappeared and two new peaks at 310 and 385 nm can be observed (Fig. S7). As we know, probe C-BH can act with CN− and causes the destruction of conjugate structure which result to blue shift of Ultraviolet spectrum which was further confirmed by UV–vis titration spectra (Fig.S8). Meanwhile, the addition product of CN− and probe C-BH can act with hydroxyl group of coumarin ring which produce ESIP, and a new absorption peak at 385 nm, the color change of the solution can be observed by naked eyes. Fluorescence spectrum of probe C-BH exhibited a peak at 563 nm, the Stokes shift was calculated as 238 nm (Fig. S9), as we know, such a large Stokes shift have never reported in the field of CN− probe. Subsequently, fluorescence titration experiment was carried out for evaluating the ability of the probe to recognize CN−, as showed in Fig. 2a, the solution of the probe C-BH showed strong fluorescence at 563 nm, with the added of CN− from 0–60 μM, the fluorescence peak at 563 nm almost decreased gradually while a new peak located 440 nm was appeared and increased gradually. At 440 nm, the relationship between fluorescence intensity and CN− can be described by the following linear equation: Y = 135.39X + 777.40, R2 = 0.99 (Fig. 2b), the detection limit (3σ) was determined to be 0.05 μM (S/N = 3). At 563 nm, the relationship between fluorescence intensity and CN− can be described by the logarithmic equation Y = 1865.98−384.94ln (x-3.88), R2 = 0.95 (Fig. 2c). In addition, the relationship between
CN\(^-\) concentration and \(I_{440\text{ nm}} / I_{563\text{ nm}}\) ratio was further to be calculated and the result was showed in Fig. 2d, the ratio of \(I_{440\text{ nm}}\) to \(I_{563\text{ nm}}\) showed a good linear relationship in the concentration range of 0–60 \(\mu\text{M}\), Y = 0.3374X - 0.7975, \(R^2 = 0.99\). These data fully confirm that probe C-BH can detect CN\(^-\) effectively with a good linear relationship. Compared with reported probes, C-BH showed great advantages (Table 1) [4, 5, 30–32] which can be applied to the detection of trace CN\(^-\) with high sensitive.

**Selectivity**

Considering that the ultimate application of probe is biological system, it is necessary to evaluate the effect of different interferences in biological system. Herein, various spices such as Cu\(^{2+}\), Ni\(^{2+}\), Fe\(^{3+}\), GSH, Hcy, Cys, H\(_2\)O\(_2\), CH\(_3\)COO\(^-\), Cl\(^-\), S\(^2-\), NO\(_2^-\), S\(_2\)O\(_3^-\), HSO\(_4^-\) (60 \(\mu\text{M}\)) were assessed under the same test conditions. The result was showed in Fig. 3, only CN\(^-\) can trigger violent fluorescence change while none of the selected species candidates caused obvious changes which demonstrated that our probe C-BH exhibited higher selectivity and reliability towards CN\(^-\) detection in complex organisms.

**The Response Time and pH of C-BH to CN\(^-\)**

The time dependence of the fluorescence of probe C-BH toward CN\(^-\) was investigated. Free probe C-BH showed strong stability while 60 \(\mu\text{M}\) CN\(^-\) was added, the fluorescence

### Table 1 The comparison of C-BH with the reported probes for sensing of CN\(^-\)

| Probe  | Detection limit (\(\mu\text{M}\)) | Cell imaging | Ref.   |
|--------|---------------------------------|--------------|--------|
| NPC    | 0.145                           | Yes          | [4]    |
| TPA–BTD–MT | 0.087                         | Yes          | [5]    |
| P1     | 0.75                            | No           | [30]   |
| L1     | 1.1                             | No           | [31]   |
| Sensor 1 | 0.03                           | Yes          | [32]   |
| C-BH   | 0.05                            | Yes          | This work |

![Fig. 2 a Changes of fluorescence spectra of probe C-BH (10 \(\mu\text{M}\)) with titration of different concentration of CN\(^-\) (0–60 \(\mu\text{M}\)). b the relationship of \(I_{440\text{ nm}}\) versus the concentration of CN\(^-\) over the range from 0 to 60 \(\mu\text{M}\). c the relationship of \(I_{563\text{ nm}}\) versus the concentration of CN\(^-\) over the range from 0 to 60 \(\mu\text{M}\). d the relationship of \(I_{440\text{ nm}}/I_{563\text{ nm}}\) versus the concentration of CN\(^-\) over the range from 0 to 60 \(\mu\text{M}\). Ex = 370 nm](#)
intensity at 440 nm was significantly enhanced within seconds and stable in about 20 min (Fig. 4a). In order to explore the application of the probe C-BH under physiological conditions, the effect of pH on the fluorescence properties was evaluated, the result was showed in Fig. 4b, probe C-BH showed stable fluorescence in the pH range of 2–10 and C-BH-CN− showed stable fluorescence in the pH range of 3–10, indicating probe C-BH can detect CN− in the life system.

**Mechanism Description**

As we know, CN− can act with C=N bond and formation of cyano substituted products which was mentioned in the previous report [31]. In this study, the reaction mechanism can be described as follows: in the presence of CN−, the C=N bond of probe C-BH was destroyed and give cyano substituted product which was confirmed by using MS analysis data (m/z = 408.2 Fig. S10). Then 1H NMR titration spectra was also carried out and the results showed that with the addition of CN−, the Ha of C-BH was change to Hb C-BH/CN− (Fig. S11). In addition, a new point can be observed in Fig. S12 by TLC after probe C-BH acted CN−. The specific mechanism was shown in Scheme 2.

**Scheme 2** Proposed mechanism of probe C-BH for highly selective sensing of CN−
Theoretical Calculation

In order to further study the interaction mechanism between the probe C-BH and CN\(^-\). Density functional theory (DFT) calculation was performed at the B3LYP level of the Gauss09 program to understand their electronic structure. Figure 5 showed the optimized geometric configurations of C-BH and C-BH-CN and their HOMOs and LUMOs. The energy gaps between HOMOs and LUMOs of C-BH and C-BH-CN were calculated to be 1.5792 eV and 3.5178 eV respectively. In the presence of CN\(^-\), the increased energy gap between the HOMO and LUMO is responsible for the blue-shift emission, which was consistent with the experimental results of ultraviolet and fluorescence spectrum.

MTT Assay and Cellular Imaging

Better optical properties based on probe C-BH, we further explore its application in biology. First, the toxicity of C-BH against HeLa cells should be estimated by using the MTT assay. The suspension of HeLa cells cultured in a cell incubator for 24 h. various concentrations of C-BH (0, 20, 40, 60, 80 and 100 μg/mL) were added and cultured in the cell incubator for another 24 h. The results showed in Fig. S13, Hela cell viability is still around 92% after treatment with 100 μg/mL of the probe C-BH which illustrated that the toxicity of probe C-BH is very low and very suitable for cell application.

We further investigated the application of the probe for CN\(^-\) imaging in living cells. Four groups of HeLa cells were prepared. The first group of HeLa cells were pre-treated with the probe C-BH (10 μM) solution for 30 min, then cultured in the cell incubator for another 30 min, washed three times with PBS buffer solution before imaging by laser confocal microscopy. The result was illustrated in Fig. 6. HeLa cells were pre-treated with the probe C-BH showed weak green fluorescence, with the increased of CN\(^-\), the fluorescence of green channel was further weakened while the fluorescence of blue channel was appeared and gradually enhanced which implied the probe C-BH can detect CN\(^-\) by two channels increased the reliability of detection in biological system.

Zebrafish Imaging

Considering the successful application of probe C-BH in cells, in order to expand its application scope, we further explored the application of probe C-BH in vivo. Zebrafish embryos (5 days old) were chosen as the animal model. Four groups of zebrafish were prepared. The first group of zebrafish were incubated with probe C-BH (10 μM) for 0.5 h, then used for fluorescence imaging. Weak green fluorescence can be observed, while the other three groups were further interacted with various CN\(^-\) (5, 10, 15 μM) for another 0.5 h after incubation with the probe C-BH. As Fig. 7 showed, with the increase of CN\(^-\), the green fluorescence almost completely quenched, and the blue channel fluorescence appeared and gradually enhanced which indicating that our probe C-BH can be used to detect CN\(^-\) in zebrafish.

Conclusions

In summary, a novel AIE-gens fluorescent probe C-BH was designed and synthetized rationally. Probe C-BH showed good selectivity, high sensitivity and dual channel detected to CN\(^-\), the obvious fluorescence changes can be recognized by naked eyes. Due to its good optical properties, probe C-BH has been applied to the detection of CN\(^-\) in living cells and zebrafish successfully. This study provides a ratio fluorescent probe for monitoring CN\(^-\) which is helpful for the early diagnosis of diseases caused by its abnormal level.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10895-021-02817-x.

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Author Contribution YL Jiang provided the initial idea for this work; SDeng, Q Lei and JZ Cai contributed to the collection and analysis of test data; and S Deng contributed to the theoretical calculation. YL Jiang and J Shen contributed to the analyses of results; YL Jiang wrote the paper.
Data Availability  The authors declare that [the/all other] data supporting the findings of this study are available within the article [and its supplementary information files].

Code Availability  Software Used: ADF 2017.

Declarations

Ethics Approval  Zebrafish studies were approved by the Ethics Committee and IACUC of Qilu Health Science Center, Nanjing Normal University, and were conducted in compliance with European guidelines for the care and use of laboratory animals.

Conflict of Interest  The author declares no conflict interest.

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