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COMMUNICATION

A ratiometric fluorescent probe for oxalate based on alkyne-conjugated carboxamidoquinolines in aqueous solution and imaging in living cells†

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A novel ratiometric fluorescent probe for oxalic acid was designed and synthesized, based on the zinc-containing [DAQZ@2Zn^{2+}] complex. It shows highly selective "on-off" fluorescence changes with a more than 20 nm blue shift in wavelength for oxalic acids in aqueous solution. Moreover, it can fluorescently respond to oxalic acid in living cells.

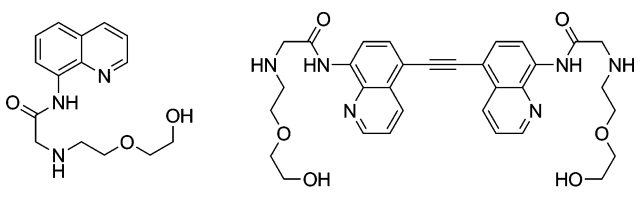
Oxalic acid is abundantly present in nature and widely used in industry, and oxalate is also an important nutrient in the human diet found in spinach, beet leaves, *etc*.[1] As is well known, oxalate is a primary chelator of calcium ions and forms chelates with dietary calcium, thus making the complex unavailable for adsorption in the body. The absorbed oxalate is precipitated as insoluble salts and finally accumulates in the renal tissue.[2] High oxalate concentrations provide conditions for precipitation of calcium oxalate crystals in the urine. Both calcium oxalate crystals and oxalate ions induce renal injury.[3] An excess amount of oxalic acid in the urine may be indicative of kidney lesions and pancreatic insufficiency.[4] Therefore, the determination of oxalate in food chemistry and in clinical analysis is of extensive significance.

Determination methods for oxalate, such as spectrophotometric, liquid and gas chromatography, amperometric and capillary electrophoresis, have been proposed.[5] Many of these methods often require complicated, multi-step samples pretreatment or need sophisticated equipment. So, to avoid tedious sample preparation and enhance detection limits, a simple and inexpensive method is required. Fluorescence assay has become a powerful optical technique for quantitative detection of analytes owing to its easily detected signals, low detection limit and the potential for real time detection in living systems with high spatial resolution.[6] In particular, during the past few years, fluorescence sensors for anions have become attractive. Among those artificial receptors for anions, most of them bind analytes either by hydrogen-bonding or metal–ligand interactions. It is known that metal–ligand interactions are stronger than H-bond interactions and compete successfully with water for the anions, thus allowing recognition studies to be carried out in aqueous solutions at physiological pH.[7] Some fluorescent sensors for anions have been investigated by using Zn^{2+} and Cd^{2+} containing receptors as binding centers, because of their redox inactivity and d^{10} electronic configuration which prevent any interference with the proximate fluorophore.[8]

Up to now, fluorescent probes for the detection of oxalic acid are still rare. Reported fluorescent systems include the use of a dinuclear copper(II) complex,[9] and anthrylamine functionalized with the all-*cis*-2,4,6-triamino-1,3,5-trimethoxycyclohexane-zinc(II) complex.[10] But none of them showed practical applications in biological systems. So, novel fluorescent chemosensors with high selectivity and sensitivity for oxalic acid in aqueous solution and living cells became our target.

Meanwhile, fluorescent sensing for the specified analyte by measuring the changes in fluorescence intensity (fluorescence quenching or enhancement) can be affected by many other variable factors such as the concentration of the sensor, the environment around the sensor molecule (pH, polarity, temperature, and so forth). However, ratiometric fluorescent sensors permit measuring the changes in the ratio of the signal intensities, which increase the dynamic range and provide built-in correction for environmental effects.[11]

Herein, following our interest in the development of novel carboxamidoquinoline systems, we designed and synthesized a simple sensor, **DAQZ**, based on reported fluorescent sensor **AQZ** (Scheme 1).[12] By virtue of the extended π-system of carboxamidoquinoline through an alkyne group, **DAQZ** is highly fluorescent with longer absorbance wavelength than **AQZ**. More importantly, once **DAQZ** binds the target species, the two carboxamidoquinoline are turned to the "alive" state simultaneously, so it has more significant signal changes than the corresponding chemosensor **AQZ** with one fluorophore.



Scheme 1 Structures of the two fluorescent sensors.

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† Electronic supplementary information (ESI) available: Scheme S1 and Fig. S1–S12. See DOI: 10.1039/c0dt01364a

The target compound, **DAQZ**, was synthesized through the corresponding 5,5′-(ethyne-1,2-diyl)diquinolin-8-amine intermediate **3**, which was obtained by coupling 5-iodoquinolin-8-amine (**1**) with 5-ethynylquinolin-8-amine (**2**) by the Castro–Stephens/Sonogashira protocol (ESI, Scheme S1†).[13]

The [**DAQZ@2Zn**$^{2+}$] complex was prepared by titrating Zn^{2+} with a solution of **DAQZ** in a tris-HCl solution (ethanol–water, 1 : 9, v/v). The result was characterized by UV-vis absorption spectra and fluorescence emission spectra. As shown in Fig. S1,† **DAQZ** has a main absorption peak centered at 375 nm in ethanol–water (1 : 9, v/v) solution, which is longer than **AQZ** over 50 nm. While adding Zn^{2+} to the solution of **DAZQ**, a new absorption peak at 426 nm appeared, and the peak at 375 nm decreased, with an isosbestic point at 400 nm (ESI, Fig. S1†).

The emission spectrum of free **DAQZ** displays a broad band with a maximum at 470 nm in an aqueous tris-HCl buffer solution (Fig. 1). When Zn^{2+} was added to the solution of **DAQZ**, a 42 nm red-shifted band was observed with a significant emission decrease and increase at 470 nm and 512 nm, respectively, and a clear isoemission point at 487 nm, which was attributed to the formation of a [**DAQZ@2Zn**$^{2+}$] complex (Fig. 1). The inset in Fig. 1 exhibits the dependence of the intensity ratios of emission at 512 nm and 470 nm ($I_{512\,nm}/I_{470\,nm}$) on Zn^{2+}, which indicates the formation of a [**DAQZ@2Zn**$^{2+}$] adduct of 1 : 2 stoichiometry. Moreover, a Job's plot, which exhibits a maximum at 0.667 fraction of Zn^{2+}, further indicates that only a 1 : 2 complex is formed (ESI, Fig. S2†). In order to determine a reliable association constant, a more diluted **DAQZ** (0.2 μM) solution was titrated with Zn^{2+} to get a smoother titration curve. And the association constant is determined to be $K_{s1} = 1.9 \times 10^5$, $K_{s2} = 9.2 \times 10^5$ from this titration curve by a non-linear least-squares analysis (ESI, Fig. S3†). The Φ_F values of free **DAQZ** and the [**DAQZ@2Zn**$^{2+}$] complex are 0.18 and 0.21, respectively.[14]

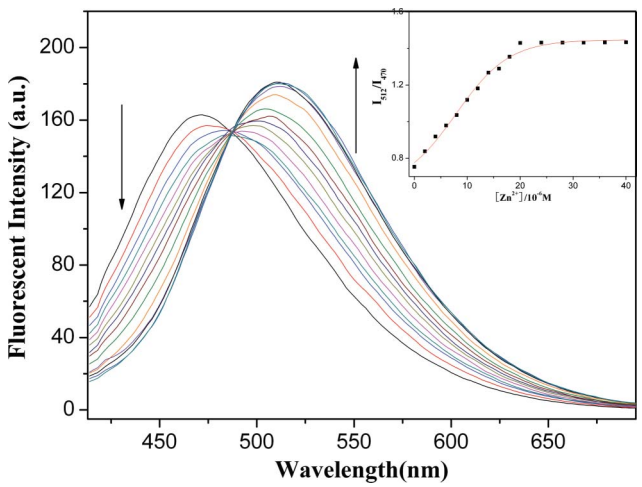

Fig. 1 Emission spectra of a solution of **DAQZ** (10 μM) in the presence of increasing Zn^{2+} concentration (0–4 equiv.) in tris-HCl (0.01 M) solution (ethanol–water, 1 : 9, pH = 6.02) Inset: ratiometric calibration curve $I_{512\,nm}/I_{470\,nm}$ as a function of Zn^{2+} concentration (λ_{ex} = 400 nm).

As emphasized above, it is of particular interest to explore the possibility of the [**DAQZ@2Zn**$^{2+}$] complex for effectively recognizing the presence of oxalic acid. The influence of pH on the fluorescence intensity of the [**DAQZ@2Zn**$^{2+}$] complex was investigated and the result is shown in the ESI, Fig. S4†. The emission intensity of the [**DAQZ@2Zn**$^{2+}$] complex had no obvious change in the range of pH 6.0–8.0. Therefore, all the detections of various mono- and dicarboxylic acids were evaluated in the tris-HCl buffer solutions (10 mM, ethanol–water, 1 : 9, v/v) at pH 7.02.

The [**DAQZ@2Zn**$^{2+}$] complex was prepared by adding 6.0 μM Zn^{2+} to a tris-HCl solution of **DAQZ** (3 μM) for the detection of oxalic acid. Fig. 2 displays the changes profile of emission spectra of the [**DAQZ@2Zn**$^{2+}$] complex with oxalic acid concentration at pH = 7.02 (10 mM, tris-HCl). With the increasing of oxalic acid concentration in a solution of the [**DAQZ@2Zn**$^{2+}$] complex, the fluorescence intensity of the [**DAQZ@2Zn**$^{2+}$] complex sharply decreased with a 20 nm blue shift in wavelength. The fluorescent intensity ratio at 467 nm and 513 nm ($I_{467\,nm}/I_{513\,nm}$) increased in a linear fashion with the concentration of 0–120 μM of oxalic acid (linearly dependent coefficient: R^2 = 0.9987, Fig. 3a). This indicated that the [**DAQZ@2Zn**$^{2+}$] complex can be potentially used to quantitatively detect oxalic acid concentration. The Job's plot indicates that a 1 : 2 complex is formed between the [**DAQZ@2Zn**$^{2+}$] complex and oxalic acid (ESI, Fig. S5†). The binding constant for oxalic acid was calculated to be $K_{s1} = 2.6 \times 10^4$, $K_{s2} = 2.5 \times 10^3$ through a least-squares analysis of titration profiles (ESI, Fig. S6†). ^{1}H-NMR studies also provided evidence for the interaction between the [**DAQZ@2Zn**$^{2+}$] complex and oxalate (ESI, Fig.S7–S9†). Upon addition of 2 equiv. of Zn^{2+} in the DAQZ, the peaks (Fig. S7,† from 2.8 ppm to 3.7 ppm) assigned to methylene protons of the alkoxyethylamino chain shifted significantly downfield and broadened (Fig. S8,† from 3.4 ppm to 4.3 ppm). Obviously, N–Zn(II) and O–Zn(II) (N,O-alkoxyethylamino chain) complexation lowers the electron density of the N/O and the deshielding effect is responsible for these NMR spectral changes. Then, after addition of 2 equiv. of oxalate in the [**DAZQ@2Zn**$^{2+}$] complex, the peaks shifted moderately upfield (Fig. S9,† from 3.3 ppm to 4.2 ppm). It indicates that the interaction of N–Zn(II) and O–Zn(II) are weakened and the shielding effect is recovered a little bit after oxalate binding the

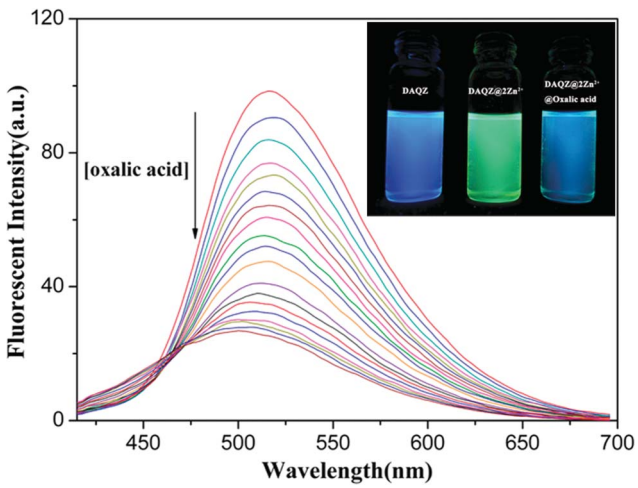

Fig. 2 Oxalic acid titration induced the fluorescence spectra changes of the [**DAQZ@2Zn**$^{2+}$] complex (3 μM) in tris-HCl (0.01 M) solution (ethanol–water, 1 : 9, pH = 7.02, λ_{ex} = 400 nm). Inset: images were taken under UV irradiation. Left: **DAQZ**, middle: [**DAQZ@2Zn**$^{2+}$], right: [**DAQZ@2Zn**$^{2+}$**@oxalic acid**].

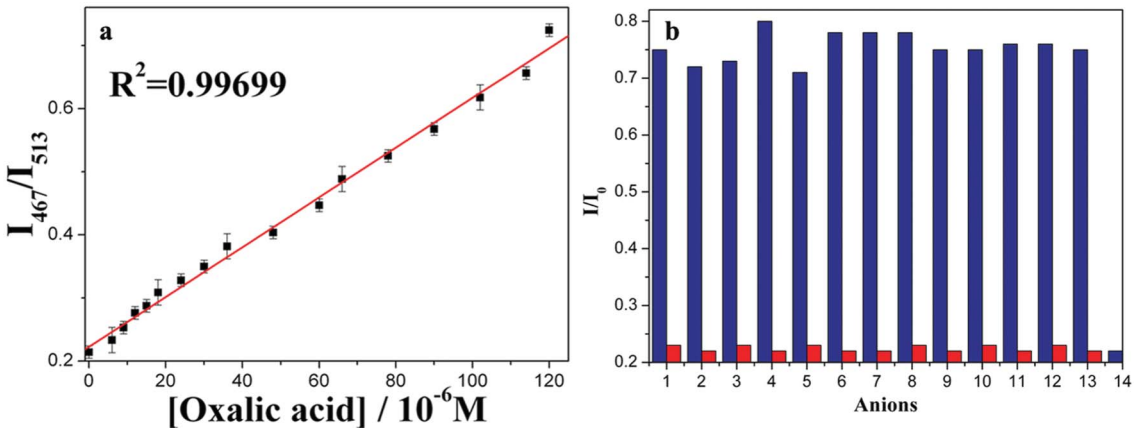

Fig. 3 (a) Ratiometric calibration curve $I_{467\,nm}/I_{513\,nm}$ as a function of oxalic acid concentration in tris-HCl (0.01 M) solution ([**DAQZ@2Zn²⁺**] complex = 3 μM, [oxalic acid] = 0–120 μM). (b) Fluorescence response of the [**DAQZ@2Zn²⁺**] complex (3 μM) in tris-HCl (0.01 M, ethanol–water, 1 : 9, pH = 7.02) solution with different mono-, dicarboxylic acids and phosphate anions ([anions] = 120 μM). Blue bars represent the response of the [DAQZ@2Zn²⁺] complex in the presence of the appropriate anion of interest. Red bars represent the response upon addition of oxalic acid to a solution of the [DAQZ@2Zn²⁺] complex and the appropriate anion of interest. (1) formic acid, (2) acetic acid, (3) propionic acid, (4) butanoic acids, (5) malonic acid, (6) succinic acid, (7) adipic acid, (8) sebacic acid, (9) *o*-phthalic acid, (10) *p*-phthalic acid, (11) PO_4^{3-}, (12) HPO_4^{2-}, (13) $H_2PO_4^-$, (14) oxalic acid.

Zn(II). The quenching of fluorescence may result from the binding to Zn^{2+} in the [**DAQZ@2Zn²⁺**] complex by oxalic acid as a chelating ligand, which weakens the ICT process from the nitrogen atom of the heterocycle to the metal ion.

To test the metal–ligand selectivity, we titrated the [**DAQZ@2Zn²⁺**] complex solutions with other mono-, dicarboxylic acids and phosphate anions. The results are shown in Fig. 3b, only a decrease of about 20% was observed in the emission spectra after addition of various mono- and dicarboxylic acids such as formic acid, acetic acid, propionic acid, butanoic acids, malonic acid, succinic acid, adipic acid, sebacic acid, *o*-phthalic acid and *p*-phthalic acid, and phosphate anions such as PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$. However, under identical conditions, the addition of oxalic acid led to an 80% decrease of the emission intensity and a 20 nm blue-shift in wavelength. The competition experiments for the [**DAQZ@2Zn²⁺**] complex were also conducted (Fig. 3b). When the same amount of oxalic acid was added into the solution of [**DAQZ@2Zn²⁺**] complex in the presence of other mono-, dicarboxylic acids and phosphate anions, nearly the same decrease of the emission intensity was observed by comparison with that of oxalic acid only. More importantly, if the amount of oxalic acid needed to decrease the initial fluorescence emission by 12% is taken as a reference detection limit, an oxalic acid concentration down to 3.0 μM was able to be measured (ESI, Fig. S10†), which is much lower than that measured by the reported oxalic acid anion receptors.[9–10] The results indicated the [**DAQZ@2Zn²⁺**] complex used as receptor could clearly discriminate oxalic acid from the other mono-, dicarboxylic acids and phosphate anions with high sensitivity in aqueous solutions at neutral pH conditions.

We further demonstrate practical application of the [**DAQZ@2Zn²⁺**] complex to cultured living cells (HeLa cells). Incubation of HeLa cells with the [**DAQZ@2Zn²⁺**] complex for 1.0 h at 37 °C was followed by the addition of oxalic acid and then was incubated for another 0.5 h. The quenching of fluorescence was observed (Fig. 4). And no significant quenching of fluorescence was observed when the cells were cultured with other different

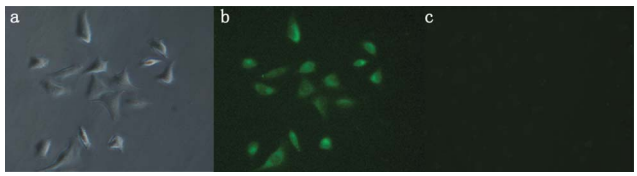

Fig. 4 Fluorescence images of the [**DAQZ@2Zn²⁺**] complex ([**DAQZ**] = 4.0 μM) induced by intracellular oxalic acid in HeLa cells. (a) Bright-field transmission image of HeLa Cells incubated with the [**DAQZ@2Zn²⁺**] complex. (b) Fluorescence image of HeLa cells incubated with the [**DAQZ@2Zn²⁺**] complex. (c) Fluorescence image of HeLa cells incubated with the [**DAQZ@2Zn²⁺**] complex for 60 min, washed three times, and then further incubated with 1×10^{-4} M oxalic acid for 30 min.

kinds of mono-, dicarboxylic acids (ESI, Fig. S11†). The results suggest that the [**DAQZ@2Zn²⁺**] complex can penetrate the cell membrane and can be used for imaging oxalic acid in living cells.

In summary, a novel class of fluorescent [**DAQZ@2Zn²⁺**] complex was designed and synthesized. Among common mono-carboxylic acids, long-chain dicarboxylic acids and phosphate anions, it specifically responded to the presence of oxalic acid with high sensitivity in aqueous solution. Moreover, the living cell image experiments further demonstrate the possibility of application in biological systems.

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