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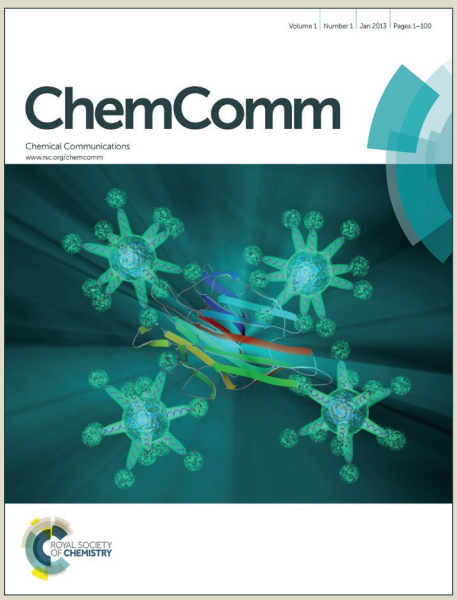

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View Article Online
DOI: 10.1039/C5CC09092J

Journal Name

COMMUNICATION

A mitochondria-targeted fluorescent probe for ratiometric detection of endogenous sulfur dioxide derivatives in cancer cells

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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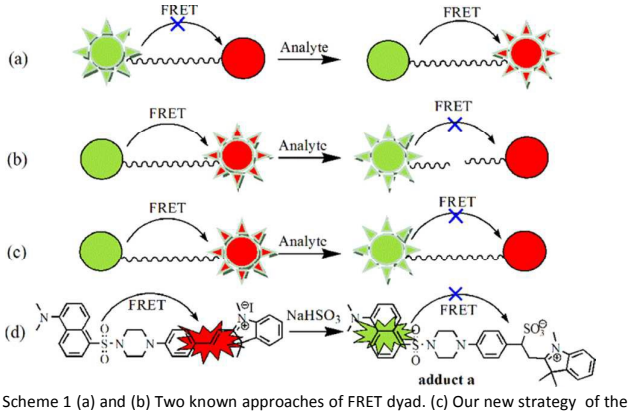
A new mitochondria-targeted fluorescent probe HCy-D, constructed by dansyl and hemicyanine fluorophore, for SO_2 derivatives (HSO_3^-/SO_3^{2-}) was presented. This probe was designed based on a new FRET platform. HCy-D showed a ratiometric, sensitive and rapid response toward HSO_3^-/SO_3^{2-}. Importantly, HCy-D was successfully used to fluorescence imaging of endogenous bisulfite in HepG2 cells, which may benefit cancer diagnosis by discriminating liver cancer cells from normal liver cells.

Sulfur dioxide (SO_2) is a main air pollutant which exists as sulfite (SO_3^{2-}) and bisulfite (HSO_3^-) anions in aqueous solution. However, SO_2 can be produced enzymatically in cytosols and mitochondria of cells during decomposition of sulfur-containing amino acids.[1] Like nitric oxide (NO), SO_2 is a newly recognized gaseous signal transmitter involved in many physiological processes (such as regulation of vascular smooth muscle tone and lowering blood pressure).[2] SO_2 may serve as antioxidant and in recent years, a few favourable redox-responsive fluorescent probes have been developed and applied to explore the generation, transport, physiological function, and pathogenic mechanisms of reactive oxygen species and antioxidants.[3] Nonetheless, the biological roles of SO_2 still remain largely unknown. The detection of SO_2 in cells is a bottleneck problem.

Ratiometric fluorescent probes can detect trace amounts of analyte accurately by built-in correction with two emission bands and eliminating the environmental impacts.[4] Till now, a few well-behaved ratiometric fluorescent probes for HSO_3^-/SO_3^{2-} have been developed.[5] Even so, these probes showed some drawbacks such as unsatisfactory detection limits,[5a-d,5g,5n] long response time,[5a-d,5f,5i,5p,5q,5s] inferior selectivity over H_2S,[5g,5j,5p] ultraviolet light excitation[5b-e,5k,5r] or two excitation wavelengths involved.[5h,5m,5o,5s] Thus, still a big room for improvement is there.

Förster resonance energy transfer (FRET) has been widely used to construct ratiometric fluorescent probes, in which nonradiative energy transfers from an excited dye donor to a dye acceptor in the ground state.[6] Generally, the FRET process on/off is controlled by modulation of the acceptor molar absorption coefficient (Scheme 1a) or tuning the donor-acceptor distance (Scheme 1b).[6a,6c] Here a new strategy to construct ratiometric fluorescent probes based on FRET was introduced. The FRET process may occur in the dyad to prohibit the donor fluorescence and to enhance the acceptor fluorescence. While upon reaction with analyte, the FRET process will be cancelled because of the changed absorption band of the acceptor to restore the donor fluorescence (Scheme 1c). Thus, a new ratiometric fluorescent probe was designed (Scheme 1d). A dansyl fluorophore was selected as the donor for its good spectroscopic properties.[7] A hemicyanine fluorophore was chosen as the acceptor because it may not only serve as the quencher of donor fluorescence, but also serve as HSO_3^-/SO_3^{2-} receptor.[5f,5h,5j-m] Importantly, fluorescence emission band of the donor and UV-vis absorption band of the acceptor overlap fully.



Scheme 1 (a) and (b) Two known approaches of FRET dyad. (c) Our new strategy of the FRET dyad toward bisulfite. (d) Modulation of the FRET in probe HCy-D. Green : donor; Red: acceptor.

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† Electronic Supplementary Information (ESI) available: Experimental details, original NMR, MS spectra and supplementary Figs. See DOI: 10.1039/x0xx00000x

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DOI: 10.1039/C5CC09092J

Probe HCy-D was synthesized by two steps with good yield (Scheme S1, ESI†). The structure was fully characterized by IR, ^{1}H NMR, ^{13}C NMR and HRMS (see ESI). The response to HSO_3^-/SO_3^{2-} was investigated in PBS (10 mM, pH = 7.4, containing 30% DMF). $NaHSO_3$ was used as source of HSO_3^-/SO_3^{2-} since HSO_3^- and SO_3^{2-} coexisted in neutral aqueous solution with a molar ratio of about 3:1. The red-color solution of HCy-D became colorless upon addition of 10 equiv. of $NaHSO_3$ under visible light. Correspondingly, the red fluorescence changed into green at 365 nm irradiation (Fig. 1a). Thus HCy-D showed great potential in colormetric and ratiometric fluorescence detection of HSO_3^-/SO_3^{2-}. Subsequently, time-dependent fluorescence response to HSO_3^-/SO_3^{2-} was examined (Fig. 1b, and Fig. S1, ESI†), which showed that the reaction could be completed in 2 min, and I_{530}/I_{582} (the intensity ratio of two emission bands at 530 nm and 582 nm) peaked and stayed unchanged in 1 h. Such a rapid response is among the fastest ones (Table S1, ESI†), which is appealing for real-time detection. The influence of solution pH was also investigated (Fig. 1c). Probe HCy-D itself is stable in the range of pH 4 to 8.3. After addition of 6 equiv. of $NaHSO_3$, I_{530}/I_{582} increased greatly and peaked from pH 6 to 8.3, implying that HCy-D could be used in a wide pH range including physiological conditions.

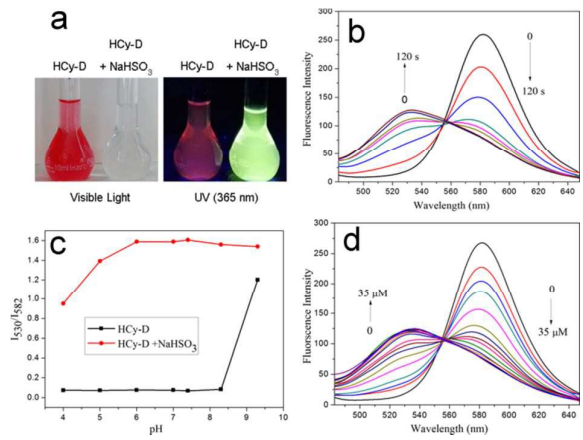

Fig. 1 (a) Color change of HCy-D solution (10 μM, in 10 mM PBS containing 30% DMF) in the absence or presence of 10 equiv. $NaHSO_3$. (b) Time-dependent fluorescence spectra of HCy-D (5 μM) in the presence of 6 equiv. of $NaHSO_3$. (c) pH-Dependent fluorescence spectra of HCy-D (5 μM) in the absence or presence of 6 equiv. of $NaHSO_3$. (d) Fluorescence titration spectra of HCy-D (5 μM) upon addition of $NaHSO_3$ (0-35 μM). λex = 410 nm, slit: 10 nm/12 nm.

Upon addition of $NaHSO_3$ (0-7 equiv.) to the buffered solution of probe HCy-D, a fluorescence titration curve was obtained (Fig. 1d). The characteristic emission band of the probe (centered at 582 nm) decreased gradually with a new emission band (centered at 530 nm) emerged and increased. A 23-fold enhancement of the fluorescence ratio I_{530}/I_{582} was observed when about 6 equiv. of $NaHSO_3$ was added (Fig. S2, ESI†). This proved the high reaction efficiency between HCy-D and HSO_3^-/SO_3^{2-}, compared with more than 10 equiv. of $NaHSO_3$ or Na_2SO_3 needed for some reported probes.[5g-i,5l,5n,5q] I_{530}/I_{582} was good linearly proportional to the concentrations of $NaHSO_3$ over a range of 0-15 μM, and the detection limit was calculated to be 0.1 μM (S/N = 3, Fig. S3, ESI†), which was superior to some reported probes[5f,5g,5p,5r] and sensitive enough to quantitatively detect endogenously produced bisulfite[5v,5w,8]

Hemicyanine fluorophore was reported to be able to capture nucleophilic agents via 1,2- or 1,4- addition.[5f,9] To shed light on the reaction mechanism, two compounds, Donor and Acceptor (Scheme S1, ESI†), were synthesized and their reactivity toward HSO_3^-/SO_3^{2-} was examined. The fluorescence of the Acceptor was quenched in the presence of 20 equiv. of $NaHSO_3$, whereas that of the donor was unaffected (Fig. S4, ESI†). What's more, upon addition of $NaHSO_3$ to the buffered solution of probe HCy-D, the absorption band peaked at 508 nm decreased gradually (Fig. S5, ESI†) and the color of the probe solution changed from red to colorless (Fig. 1a), indicating the interruption of the conjugated structure in HCy-D. Thus the hemicyanine fluorophore of HCy-D might be responsible for the reaction between probe HCy-D and HSO_3^-/SO_3^{2-}.

Furthermore, the addition was confirmed by HRMS in which two dominant peaks at m/z 579.2951 and 661.2666 were attributed to probe HCy-D and $[HCy\text{-}D + H_2SO_3]^+$, respectively (Fig. S6, ESI†). This strongly suggested the formation of a 1:1 addition product. Then ^{1}H NMR titration was performed in DMSO-d6 (containing 20% D_2O) (Scheme S2c, ESI†). The proton shifts of H1 and H2 disappeared which indicated that 1,4-addition instead of 1,2-addition was involved in the reaction. What's more, two sets of proton signals were presented in the spectrum. According to literatures,[5h,5m,5j,5k,5t] we attributed the two sets of signals to compounds **adduct a** and **adduct b**, respectively. We attempted to isolate the two products but failed due to the starting material (HCy-D) would be retrieved upon purification, even though the products are stable in reaction solution. To clarify the reaction mechanism involved, a clean spectrum (Scheme S2d, ESI†) was got in DMSO-d6 (containing 80% D_2O) which could be assigned to **adduct a** and used to compare with the spectrum that presented two sets of proton signals. Based on above findings, we deduced that the ratio of **adduct a** and **adduct b** may be influenced by the content of water. The proposed mechanism was presented in Scheme S2a.[5h] Notably, the fluorescence change of the probe when it responded to HSO_3^-/SO_3^{2-} will not be distinguished by **adduct a** or **adduct b** because the conjugation system in hemicyanine fluorophore was undoubtedly blocked. The fluorescence of **adduct a** or **b** is dominated by the dansyl fluorophore, whose structure was unaffected by the reaction.

The fluorescence emission band of the Donor had a high overlap level with the absorption band of the Acceptor (Fig. S7, ESI†). The energy transfer efficiency from the Donor to the Acceptor was calculated to be 95%. So for HCy-D, the FRET will proceed from the dansyl fluorophore to the hemicyanine fluorophore once the energy donor is excited, which could weaken or even quench the fluorescence of the dansyl fluorophore. However, addition of $NaHSO_3$ will interrupt the π-conjugation system in the hemicyanine fluorophore to cancel the FRET process due to the low overlap level (Fig. S7a, ESI†) and restore the fluorescence emission of the dansyl structure. What's more, both probe HCy-D and the Donor can be

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excited at 410 nm but the Acceptor cannot, which further confirms the FRET process in probe HCy-D (Fig. S7b, ESI†) Theoretical calculation methods have been powerful tools for many chemical processes.[10] Recently, theoretical calculations have been applied to discuss the sensing mechanism of ICT-based fluorescent probes.[5i,5l] The sensing mechanism of probe HCy-D toward bisulfite could also be rationalized by theoretical calculations. The results are consistent with reported probes (Scheme S3 and Table S2).[5i,5l] The FRET process was also verified by theoretical calculations with Gaussian09 (Fig. S8). Compared to most reported ratiometric probes designed on modulation of the ICT process alone (Table S1, ESI†), our probe for HSO_3^-/SO_3^{2-} was based on a special FRET-ICT (Scheme S4, ESI†) process.

Selectivity of HCy-D toward HSO_3^-/SO_3^{2-} over various biological relevant anions and small molecules was investigated (Fig. S9 and S10, ESI†). Only HSO_3^- and SO_3^{2-} led to a big enhancement of I_{530}/I_{582}, while others hardly brought about any change to I_{530}/I_{582}. The excellent selectivity for HSO_3^-/SO_3^{2-} is desirable, considering the fact that hemicyanine fluorophores can also be attacked by H_2S or thiols. [9,11] What's more, the response of HCy-D to $NaHSO_3$ was almost unaffected among potential competitive species (Fig. S11, ESI†), even in the presence of H_2S or biological thiols.

Now that probe HCy-D had shown a good optical response to HSO_3^-/SO_3^{2-} in vitro, cell imaging with HCy-D was first investigated in HeLa cells. HCy-D was photostable in living cells (Fig. S12, ESI†) and with negligible cytotoxicity at a concentration of 1-10 μM (Fig. S13, ESI†). As cationic cyanine dyes may accumulate in mitochondria,[5k,5l,9] a colocalization assay with Mito Tracker Deep Red (a mitochondrial dye) and HCy-D was conducted (Fig. 2). The fluorescence of HCy-D overlaid very well with that of Mito Tracker Deep Red and an overlap coefficient was calculated to be 0.94 (Fig. 2d), thereby implying a preferential distribution of HCy-D in mitochondria.

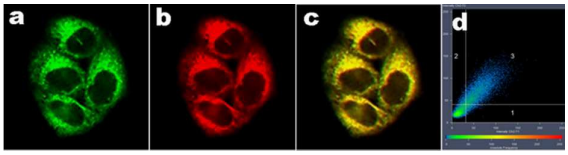

Fig. 2 HeLa cells were incubated with HCy-D (1 μM) for 1 h, followed by Mito Tracker Deep Red (1 μM) for 0.5 h. (a) The fluorescence image of HCy-D. λex = 405 nm, λem = 560-640 nm. The red fluorescence was colored as green for discrimination. (b) The fluorescence image of Mito Tracker Deep Red. λex = 639 nm, λem = 640-700 nm. (c) Merged images of (a) and (b). (d) Colocalization coefficient (Pearson's coefficient) of HCy-D and Mito Tracker Deep Red was 0.94.

Then HCy-D was applied to image HSO_3^-/SO_3^{2-} in living Hela cells. After Hela cells were incubated with HCy-D (1 μM) for 1 h, strong fluorescence in the red channel and weak fluorescence in the green channel were observed (Fig. S14, ESI†). Further incubated with different concentrations of $NaHSO_3$ for 0.5 h, the cells displayed a fluorescence decrease in the red channel and a fluorescence increase in the green channel. The results demonstrated a ratiometric and dose-dependent response of probe HCy-D toward HSO_3^-/SO_3^{2-} in living cells (Fig. S14b, ESI†).

For a further biological study, we investigated whether the probe could be used for the detection of endogenously produced bisulfite inside cells. Thiosulfate sulphurtransferase (TST) is a widespread enzyme in nature which commonly localizes in mitochondrial membrane and mitochondrial matrix.[12] Biological bisulfite can be produced endogenously from thiosulfate via TST.[13] As TST is abundant in mammalian livers,[13] HepG2 cells (human liver cancer cells) and L-02 cells (human normal liver cells) were selected to evaluate the potential of probe HCy-D for endogenous bisulfite detection.

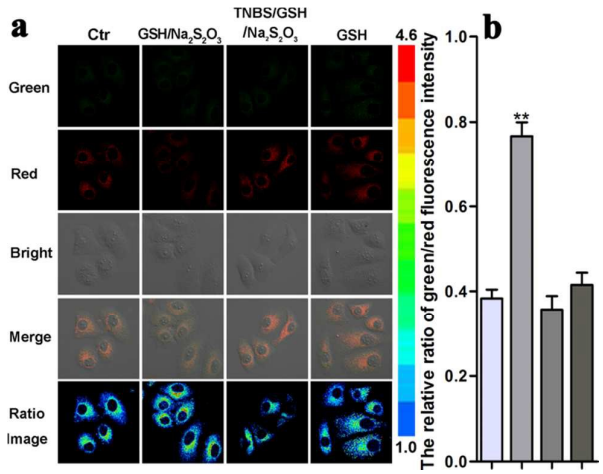

Fig. 3 (a) The first row (vertically): HepG2 cells were incubated with HCy-D (1 μM) for 1 h; The second row: HepG2 cells were incubated with HCy-D (1 μM) for 1 h, and then with 500 μM GSH and 250 μM $Na_2S_2O_3$ for 0.5 h; The third row: HepG2 cells were incubated with HCy-D (1 μM) for 1 h, then with 10 mM TNBS for 0.5 h, followed by 500 μM GSH and 250 μM $Na_2S_2O_3$ for another 0.5 h; The fourth row: HepG2 cells were incubated with HCy-D (1 μM) for 1 h, then with 500 μM GSH for another 0.5 h. (b) From left to right: the relative ratio of green/red fluorescence intensity of row 1, 2, 3 and 4 in (a). The ratio images were all obtained as F_{green}/F_{red}. Images were acquired from 405-555 nm for green fluorescence, and from 560-700 nm for red fluorescence. λex = 405 nm.

HepG2 cells were incubated with probe HCy-D (1 μM) for 1 h, washed with PBS buffer, and further incubated with 500 μM GSH and 250 μM $Na_2S_2O_3$ for 0.5 h before the images were taken. A clear fluorescence change was observed in HepG2 cells (Fig. 3). By contrast, if HCy-D loaded HepG2 cells were just incubated with GSH, no significant fluorescence change was observed. Moreover, HCy-D loaded HepG2 cells were pre-treated with 10 mM TNBS (2,4,6-trinitrobenzenesulphonate, known as a TST inhibitor) and then were incubated with 500 μM GSH and 250 μM $Na_2S_2O_3$ for 0.5 h, still no significant fluorescence change was observed (Fig. 3). These results demonstrated that the probe was capable of detecting endogenous bisulfite in HepG2 cells, which was comparable to probes reported.[13]

L-02 cells were incubated with HCy-D (1 μM) and different concentrations of $NaHSO_3$ for 0.5 h to induce significant fluorescence change. However, no significant fluorescence change was observed in L-02 cells incubated with probe HCy-D (1 μM) and GSH/ $Na_2S_2O_3$ (Fig. S15). A comparison of above results demonstrated that HCy-D could potentially be applied to differentiate between liver cancer cells and normal liver

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cells on the basis of their different endogenous bisulfite levels. This may be ascribed to the different TST content or activity, different membrane permeability of GSH or $S_2O_3^{2-}$ for these two kinds of cells or other reasons. Whatever, the new way may be of prognostic significance at cellular level. Present cancer diagnostic methods such as magnetic resonance imaging (MRI), ultrasound, positron emission tomography (PET) imaging, X-ray imaging exhibit drawbacks including limited spatial resolution, high instrument cost and radiological hazards.[14] Besides, these methods are often not effective until the middle and last stages of cancer.[15] However, this new, easy-operating method put forward here for the recognition of liver cancer cells was specific, for relatively abundant TST was reported only in liver tissues. Our method was more straightforward because most presented methods for cancer diagnosis are based on the specific recognition of intracellular or extracellular biomarkers (e.g. over-expressed enzymes).[16] We anticipate that our method could be applied to liver cancer related researches in the near future.

In summary, a ratiometric fluorescent probe based on a new FRET platform was developed for rapid, sensitive and selective detection of HSO_3^-/SO_3^{2-}. The ratiometric response was based on the π–conjugation interruption reaction along with cancelation of a FRET process. We anticipate that this sensing system should pave a new way for designing ratiometric fluorescent probes. The probe was mitochondria-targeted and was successfully applied to detect endogenous bisulfite in ratiometric and dose-dependent manner, which may be helpful to study biological roles of HSO_3^-/SO_3^{2-}. Moreover, the probe can discriminate liver cancer cells from normal liver cells at cellular level, which may be of prognostic significance.

This study was supported by the Natural Science Foundation of Shandong Province (ZR2014BM004), Major Subject of Shandong Province (2015ZDJS04003) and the National Natural Science Foundation of China (91313303).

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