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A near-infrared fluorescent probe for sensitive detection and imaging of sulfane sulfur in living cells and *in vivo*†

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Sulfane sulfur refers to ionized sulfur that is reversibly attached to other sulfur atoms in the form of 6-valence electrons (S^0). Sulfane sulfur possesses stronger nucleophilicity and reducibility than hydrogen sulfide in a series of physiological reactions, which probably makes sulfane sulfur the actual signal molecule in cells. Herein, we designed and synthesized a near-infrared (NIR) fluorescent probe BD-diSH for sensitive detection and imaging of sulfane sulfur in living cells and *in vivo*. The probe BD-diSH is composed of two moieties: the fluorophore azo-BODIPY and the sulfane sulfur recognition unit, *viz.*, 2-mercapto benzoate. BD-diSH displayed high sensitivity and selectivity towards sulfane sulfur. The mercapto group ($-SH$) of 2-mercapto benzoic acid can nucleophilically capture the sulfur atom of thiosulfoxide tautomers in sulfane sulfur to form $-SSH$. The group $-SSH$ will immediately induce intramolecular cyclization reaction and release the azo-BODIPY fluorophore to emit NIR fluorescence. The probe BD-diSH was successfully applied to detect and image sulfane sulfur in the cytoplasm of the living cells. The results illustrated that the endogenous and exogenous sulfane sulfur level changed depending on different cell lines. BD-diSH was also capable of imaging the level changes of sulfane sulfur in mice. The above applications make our new probe a potential chemical tool for the study of physiological and pathological functions of sulfur sulfide in living cells and *in vivo*.

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1. Introduction

Reactive sulfur species (RSS) is a class of sulfur-containing species, which includes hydrogen sulfide (H_2S), biothiols (RSH), S-modified cysteine adducts, sulfane sulfur as well as inorganic sulfur derivatives.¹ RSS usually functions as the anti-oxidant and signaling molecules in living cells and also plays a significant role in a variety of physical activities.² For instance, H_2S has been considered as the third gasotransmitter, which can regulate vascular tone, myocardial contractility, neurotransmission, and insulin secretion.³ However, the chemical mechanisms associated with its biological activities appear to require further validation because the concentration of “free” H_2S is low (submicromolar) in biological tissues and fluids.⁴ It

is reported that there exists a sulfane sulfur-labile pool in equilibrium with free H_2S and releasing H_2S in the cells.⁵ It is known that sulfane sulfur is one of the most important types of RSS, which points to the ionized sulfur and is reversibly attached to other sulfur atoms *via* of 6-valence electrons (S^0).⁶ Sulfane sulfur often presents in the form of elemental sulfur (S_8), dihydropolysulfides (H_2S_n , $n > 1$), persulfides ($R-S-S-H$), polysulfides ($R-S-S_n-S-R$, $n \geq 1$), thiosulfate ($S_nO_3^{2-}$, $n > 2$), and polythionates ($-SO_3-S_n-SO_3-$).⁷ Compared with H_2S , persulfide anion exhibits stronger nucleophilicity and reducibility, which results in a faster reaction activity in physical and pathological activities.⁵ Sulfane sulfur may be the actual signal molecule in cells and H_2S may be the final product released from the sulfane sulfur-based signal transduction.^{5a} In order to verify this hypothesis, the selective and sensitive methods for the detection of sulfane sulfur are urgently needed to be established.

The traditional method for sulfane sulfur detection is based on the reaction between sulfane sulfur and cyanide ions to form thiocyanate, which can be determined as ferric thiocyanate (Scheme 2).⁸ However, this method is a post-mortem process and not suitable for *in situ* detection in living cells and *in vivo*. Compared to other biological detection technologies, fluorescent imaging possesses unique advantages, such as

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high sensitivity, excellent selectivity, easy operation, non-invasiveness, and high spatial and temporal resolution; hence, it is favourable for detection of bioactive species in biological systems.⁹ It facilitates to apply chemical modification on the organic small molecule fluorophores. Therefore, the organic small molecule fluorescent probes have been rapidly developed for imaging of reactive bio-species in the cells and *in vivo*.¹⁰ Xian *et al.* pioneered to design the fluorescent probes for the detection of sulfane sulfur.¹¹ As far as we know, the fluorescent probes for the detection of H_2S_n have been rapidly developed.¹² Our group further developed a series of fluorescent probes for H_2S_n detection in the crosstalk between sulfane sulfur and superoxide anion.^{6,7b,13a} We also proposed a ratiometric NIR fluorescent probe for quantification of cysteine hydropersulfide (Cys-SSH) in living cells and *in vivo*.^{7a} However, sulfane sulfur is a collection of sulfur-containing biomolecules. The overall level changes of sulfane sulfur will potentially indicate important physiological and pathological processes. The design and synthesis of a fluorescent probe for the detection of sulfane sulfur are ongoing.⁴ It is pivotal to note that the fluorescence excitation and emission, which occur in the UV or visible region, can suffer from severe interferences, such as biological autofluorescence and tissue penetration.¹¹ Therefore, it is necessary to develop a fluorescent probe that features NIR absorption and emission for sulfane sulfur detection in living cells and *in vivo*.

Herein, we report a NIR fluorescent probe (BD-diSH) for the detection of overall sulfane sulfur in living cells and *in vivo*. As shown in Scheme 2, the probe BD-diSH is composed of two moieties: the NIR fluorophore *azo*-BODIPY and the response unit, *viz.*, 2-mercapto benzoate. The probe BD-diSH exhibits low cytotoxicity in our assays. It can selectively and sensitively detect sulfane sulfur with a low limit of detection of 10 nM ($3\sigma/k$) under the experimental conditions. Our results demonstrate that the concentrations of sulfane sulfur in different cell lines are inconstant. The overall levels of sulfane sulfur have a closed relationship with enzymes cystathionine γ -lyase (CSE) and cystathionine β -synthase (CBS). Moreover, the probe BD-diSH can be used for imaging of sulfane sulfur in organs and in mice.

2. Experimental

2.1. Synthesis and characterization of probe BD-diSH

BODIPY was synthesized in our laboratory according to the reported protocol.⁶ Initially, 2,2'-dithiodibenzoic acid (0.1 mmol, 30.64 mg) and thionyl dichloride (0.2 mmol, 25.724 mg) were dissolved in 30 mL anhydrous methylene chloride. Then, the mixture was heated to reflux for 3 h and evaporated with a rotary evaporator to obtain compound 1. A mixture of BODIPY (0.1 mmol, 50.0 mg), compound 1 (0.2 mmol, 68.39 mg), and triethylamine (0.25 mmol, 20.24 mg) in 20 mL anhydrous methylene chloride was stirred for 6 h at room temperature. Then, the mixture was partitioned between CH_2Cl_2 (50 mL) and H_2O (50 mL). The organic phase

was evaporated under reduced pressure. Then, the obtained products were dissolved in 30 mL ethanol. Sodium borohydride (0.1 mmol, 3.78 mg) was added and stirred for 15 min at room temperature. TLC was used to monitor the reaction until the starting material was completely consumed. The solvent was removed under vacuum and the obtained solid residue was purified through silica gel chromatography (200–300 mesh) with a gradient eluent of CH_2Cl_2 and CH_3OH (100 : 0 to 85 : 15 v/v). The solvent was removed under vacuum and the obtained solid was dissolved in CH_2Cl_2 , filtered, and evaporated to obtain the probe BD-SH as a green solid. Yield: 36.4 mg, 45.4%. ¹H NMR (500 MHz, $DMSO-D_6$) δ (ppm): 8.26 (m, 2H), 7.64–7.51 (m, 20 H), 7.24–7.15 (m, 4H), 6.87 (s, 2H), 5.03 (s, 2H). ¹³C NMR (125 MHz, $DMSO-D_6$) δ (ppm): 170.46, 155.85, 153.52, 151.68, 150.90, 140.16, 140.11, 140.91, 140.69, 137.37, 137.51, 134.30, 134.13, 133.96, 132.22, 131.37, 129.51, 129.45, 129.30, 129.20, 121.99, 120.40, 115.83, 106.78. LC-MS (ESI[−]): $C_{46}H_{30}BF_2N_3O_4S_2$ calcd 801.1739, found $[M - H]^-$ 800.1664.

2.2. Imaging in living cells and *in vivo*

Fluorescent images of the living cells were obtained using an Olympus FV1000 confocal laser-scanning microscope with an objective lens ($\times 40$). The excitation and emission wavelengths were selected as described in the paper. The cells were plated on Petri-dishes ($\Phi = 20$ mm) and allowed to adhere for 24 h before fluorescence imaging. The probe BD-diSH and the dyes were added to the culture plates, which were filled with 1 mL fresh complete medium. Fluorescent images of BALB/c mice were performed through a Bruker *In vivo* imaging system. The excitation and emission wavelengths were selected as described in the paper. The BALB/c mice (4–6 weeks old) were anesthetized prior to injection and during imaging.

2.3. Mice hippocampus imaging

The artificial cerebrospinal fluid (ACSF) was prepared with different solutions involving 124 mM NaCl, 2 mM KCl, 1.25 mM KH_2PO_4 , 26 mM $NaHCO_3$, 10 mM D-glucose, 2 mM $MgSO_4$, and 2 mM $CaCl_2$. The final pH of the ACSF is 7.4 after saturating with 95% O_2 /5% CO_2 . The 4–6 weeks old BALB/c mice were anesthetized with isoflurane in an acrylic desiccator before slicing the neck. The brains were removed and placed in a 20 mL dish with ice-cold oxygenated ACSF. The hippocampus was dissected and the brain was transferred to the vibratome. The thick transverse slices were cut to slices of 300–400 μm at a low speed (3 $\mu m s^{-1}$) and a vibration frequency of 70 Hz in ice-cold oxygenated ACSF. After transferring into a storage chamber, the slices were left undisturbed at RT for at least 1 h prior to staining.

2.4. Animal testing statement

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals and protocols were approved by the Institutional Animal Care and Use Committee in Binzhou Medical University, Yantai, China. Approval Number: No. BZ2014-102R.

Scheme 1 The synthetic steps for the probe BD-diSH.

3. Results and discussion

3.1. Design and detection mechanism of BD-diSH

As shown in Scheme 2, the design strategy of our as-fabricated probe BD-diSH was based on the most classical spectrophotometric method.⁴ Sulfane sulfur exists along with its thiosulfoxide tautomers, in which there is a sulfur atom that gets easily attacked by the nucleophilic CN^- to produce SCN^- . In addition, SCN^- can selectively react with Fe^{3+} to form a red coordination product, which can be measured using a spectrophotometric method. It is promising that this electrophilic property of sulfane sulfur can be used to design fluorescent probes for the detection of sulfane sulfur. The mercapto group ($-\text{SH}$) of thiophenol has nucleophilic ability similar to CN^- . Thiosulfoxide tautomers have been confirmed to react with this type of $-\text{SH}$ as a fluorescence switch for the detection of sulfane sulfur.^{11a} We chose *azo*-BODIPY as a fluorescence signal transducer because its NIR absorption and emission profiles could maximize tissue penetration, while minimizing the absorbance of heme in hemoglobin and myoglobin, water,

and lipids.¹³ Moreover, the symmetric structure of *azo*-BODIPY could offer two reactive sites for integrating response units, which would increase the detection limit of a single fluorescent probe. Herein, 2-mercapto benzoate was introduced as the response unit. The mercapto group ($-\text{SH}$) reacted with the sulfur atom of thiosulfoxide tautomers to produce the intermediate $-\text{SSH}$. Then, $-\text{SSH}$ underwent a fast intramolecular cyclization to release *azo*-BODIPY fluorophore immediately. This reaction triggered the fluorescence changes of our as-fabricated probe BD-diSH, which made the detection of sulfane sulfur possible. The synthesis of BD-diSH is illustrated in Scheme 1.

3.2. Spectroscopic response of BD-diSH towards sulfane sulfur

The absorption and fluorescence spectra of BD-diSH (10 μM) were examined under simulated physiological conditions (10 mM HEPES buffer, pH 7.4, 0.5% Tween 80 (Tw 80)). The milieu of cells is hydrophobic and liposoluble. Tw 80 is a non-ionic surfactant with wide applications in areas of food, pharmaceutical preparations, and cosmetics due to its effectiveness at low concentrations and relatively low toxicity. We employed 0.5% Tw 80 to simulate the hydrophobicity of the cells. As shown in Fig. 1a, the maximum absorption wavelength of BD-diSH was at 693 nm ($\epsilon_{693 \text{ nm}} = 3.05 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Na_2S_4 was added as the model source of sulfane sulfur for the subsequent tests. Upon the addition of 20 μM Na_2S_4 , the maximum absorption peak switched to 710 nm ($\epsilon_{710 \text{ nm}} = 3.70 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). The fluorescence titration of BD-diSH was performed in the presence of Na_2S_4 with a concentration range from 0 to 20 μM . As shown in Fig. 1b, the corresponding fluorescence emission profiles increased with the peaks centered at 734 nm. The quantum yields of BD-diSH increased from 0.016 to 0.29. Both excitation and emission wavelengths of BD-diSH were detected in the NIR region, which indicated

Scheme 2 Design strategy, molecular structures, and proposed reaction mechanism for sulfane sulfur detection.

Fig. 1 (a) UV-vis absorption spectra of the probe BD-diSH (10 μM) before and after the addition of 20 μM Na_2S_4 . (b) Fluorescence spectra of BD-diSH (10 μM) upon the addition of Na_2S_4 (0–20 μM). (c) The linear relationship between the fluorescence intensity at 736 nm and Na_2S_4 concentrations (0–20 μM) in 10 mM HEPES buffer (pH 7.4, 0.5% Tw 80). (d) Fluorescence responses of BD-diSH (10 μM) to biologically relevant RSS and other reductive materials. In each group, the bars represent relative responses at 736 nm of BD-diSH to the analytes: (1) blank, (2) 20 μM Na_2S_2 , (3) 20 μM Na_2S_4 , (4) 30 μM Cys-poly-sulfide, (5) 30 μM S_8 , (6) 1 mM Cys, (7) 1 mM Hcy, (8) 10 mM GSH, (9) 1 mM CysSSCys, (10) 1 mM GSSG, (11) 0.5 mM NaHS, (12) 0.5 mM $\text{Na}_2\text{S}_2\text{O}_3$, (13) 0.5 mM NaHSO_3 , and (14) 1 mM ascorbic acid. (e) Fluorescence responses of BD-diSH (10 μM) to various metal ions and anions. In each group, the bars represent relative responses at 736 nm of BD-diSH to the analytes: (1) blank, (2) 1 mM K^+ , (3) 1 mM Na^+ , (4) 1 mM Ca^{2+} , (5) 1 mM Mg^{2+} , (6) 1 mM Zn^{2+} , (7) 1 mM Cu^{2+} , (8) 1 mM Cl^- , (9) 1 mM Br^- , (10) 1 mM SO_4^{2-} , (11) 1 mM CO_3^{2-} , (12) 1 mM H_2PO_4^- , (13) 1 mM NO_3^- , and (14) 1 mM sodium citrate. All of the above data were recorded in 10 mM HEPES buffer (pH 7.4, 0.5% Tw 80) at 37 $^\circ\text{C}$ for 2 min. $\lambda_{\text{ex}} = 710$ nm. (f) Time-dependent fluorescence intensity of BD-diSH (10 μM) towards Na_2S_4 (20 μM). Na_2S_4 was added to the reaction at 3 min in 10 mM HEPES buffer (pH 7.4, 0.5% Tw 80). The reaction was measured for 15 min at 37 $^\circ\text{C}$. $\lambda_{\text{ex}} = 710$ nm, $\lambda_{\text{em}} = 736$ nm. The experiments were repeated seven times and the data were shown as mean ($\pm$ s.d.).

that our probe could greatly reduce the background interference and effectively improve the detectable sensitivity. There was a linear concentration-dependent fluorescence response between BD-diSH and Na_2S_4 ranging from 0 to 20 μM (Fig. 1c) and the equation obtained from the calibration curve was $F_{736\text{ nm}} = 2.95 \times 10^5 [\text{Na}_2\text{S}_4] \mu\text{M} + 3.89 \times 10^5$ ($r = 0.9985$). The detection limit toward Na_2S_4 was calculated to be 10 nM ($3\sigma/k$) under the experimental conditions, indicating the high sensitivity of our as-fabricated probe BD-diSH towards the detection of Na_2S_4 . These results revealed that our as-fabricated probe could be used to detect Na_2S_4 qualitatively and quantitatively.

3.3. Selectivity of BD-diSH towards sulfane sulfur

We next assessed the selectivity of BD-diSH towards sulfane sulfur upon the addition of various typical bio-relevant RSS, biological reductive materials, various metal ions, anions, reactive oxygen species (ROS), and reactive nitrogen species (RNS). The fluorescence responses to bio-relevant species were obtained at the time point of 5 min. As shown in Fig. 1d, only sulfane sulfur (representative: Na_2S_2 , Na_2S_4 , Cys-poly-sulfide, and S_8) could trigger strong fluorescence responses. Other reductive materials including Cys, Hcy, GSH, CysSSCys, GSSG,

NaHS, $\text{Na}_2\text{S}_2\text{O}_3$, NaHSO_3 , and ascorbic acid could not induce any fluorescence intensity changes. In addition, the fluorescence intensity of BD-diSH was also hardly affected by various metal ions and anions, such as K^+ , Na^+ , Ca^{2+} , Mg^{2+} , Zn^{2+} , Cu^{2+} , Cl^- , Br^- , SO_4^{2-} , CO_3^{2-} , H_2PO_4^- , NO_3^- , and sodium citrate (Fig. 1e). Moreover, ROS and RNS including $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$, $^1\text{O}_2$, OCl^- , H_2O_2 , $\text{ROO}^\cdot$, HNO , ONOO^- , NO_2^- , NO , $t\text{-BuOOH}$, MeLOOH , and CuOOH could not induce any changes in the fluorescence intensity (Fig. S1†). All of these results demonstrated that BD-diSH was highly selective for sulfane sulfur over other bio-relevant RSS, biological reductive materials, various metal ions, and anions. Clearly, our probe BD-diSH could potentially meet the requirements for the detection of sulfane sulfur in the complex biological samples.

3.4. Reaction kinetics of BD-diSH towards sulfane sulfur

Next, the reaction kinetics of BD-diSH (10 μM) towards Na_2S_4 (20 μM) was evaluated under the simulated physiological conditions (10 mM HEPES buffer, pH 7.4, 0.5% Tw 80). As shown in Fig. 1f, Na_2S_4 (20 μM) was added at the time point of 3 min. The fluorescence would rapidly increase nearly 20 times. Fluorescence intensity reached a plateau within 2 min. The intensity lasted in the next 10 min without attenuation. The

fast response of BD-diSH towards sulfane sulfur could meet the challenge that sulfane sulfur would rapidly metabolise in the biological system. In addition, the excellent photophysical properties would avoid the interference caused by photobleaching. These results demonstrated that the probe BD-diSH could be employed to detect the changes of sulfane sulfur in the living cells and *in vivo*.

3.5. Detection and sublocation in cells

Since the low cytotoxicity is one of the key criteria for the biological application of a fluorescent probe in the living cells, we evaluated the cytotoxicity of BD-diSH in RAW 264.7 cells *via* MTT assay. As illustrated in Fig. S4,[†] cell viabilities were over 90% even with the addition of 0–100 μM BD-diSH for 24 h.

These results indicated that our as-fabricated probe could provide low cytotoxicity for the biochemical tests in living cells and *in vivo*. On the basis of the high sensitivity and selectivity advantages of BD-diSH for the detection of sulfane sulfur under the simulated physiological conditions, we next explored its applicability to image sulfane sulfur in living cells utilizing laser-scanning confocal microscopy. We chose the cells in the same visual field as the testing target analyte. After being incubated with 1 μM BD-diSH at 37 $^{\circ}\text{C}$ for 15 min, the RAW264.7 cells displayed weak fluorescence imaging (Fig. 2a), exhibiting the level of endogenous sulfane sulfur. After a dosage of 5 μM Na_2S_4 was added to the tested system at 37 $^{\circ}\text{C}$ for 15 min, the strong intracellular fluorescence response was observed (Fig. 2e). The second dosage of 5 μM Na_2S_4 for

Fig. 2 Confocal fluorescence images of sulfane sulfur in RAW 264.7 cells stained with 1 μM BD-diSH (the red channel), 1 μM Calcein-AM (the green channel), and 1 $\mu\text{g mL}^{-1}$ Hoechst 33342 (the blue channel). Fluorescence collected window: red channel: $\lambda_{\text{ex}} = 635 \text{ nm}$, $\lambda_{\text{em}} = 700\text{--}800 \text{ nm}$; green channel: $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{--}550 \text{ nm}$; blue channel: $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 420\text{--}480 \text{ nm}$. (a) Cells loaded with 1 μM BD-diSH for 15 min. (b) Overlay of (a) and green channel. (c) Overlay of (a), green channel, and blue channel. (d) Overlay of (b), green channel, blue channel, and bright-field image. (e) Cells treated with 5 μM Na_2S_4 for 15 min. (f) Overlay of (e) and green channel. (g) Overlay of (e), green channel, and blue channel. (h) Overlay of (e), green channel, blue channel, and the bright-field image. (i) Cells treated with another dose of 5 μM Na_2S_4 for 15 min. (j) Overlay of (i) and green channel. (k) Overlay of (i), green channel, and blue channel. (l) Overlay of (i), green channel, blue channel, and the bright-field image. (m) 3D view of co-localization of the blue and green channels selected. (n) 3D view of co-localization of the blue and red channels selected. (o) 3D view of co-localization of the red and green channels selected. (p) Cells treated with 1 $\mu\text{g mL}^{-1}$ Hoechst 33342 for 30 min. (q) The colocalization areas of the blue and green channels selected. (r) The colocalization areas of the blue and red channels selected. (s) The colocalization areas of the red and green channels selected. (t) Histograms of average ratio intensities in (a), (e), and (i). (u) Flow cytometry analysis of (a). (v) Flow cytometry analysis of (e). (w) Flow cytometry analysis of (i). (x) Corresponding mean ratio intensity in (u), (v), and (w). The data were shown as mean ($\pm$ s.d.) ($n = 7$). The difference was analyzed by one-way ANOVA. *** $P < 0.001$ vs. (a).

15 min could induce another fluorescence burst (Fig. 2i). These results displayed that our probe not only could be used to detect endogenous sulfane sulfur, but also had a large detection capacity. To further confirm the fluorescence signal changes in the RAW 264.7 cells caused by Na_2S_4 , we performed flow cytometry assay to test and verify the above results (Fig. 2a, e and i) as flow cytometry allows rapid analysis of millions of cells and generates statistically convincing data. The two testing results were consistent with each other (Fig. 2u–w, t and x). We next performed colocalization assay to examine the intracellular location of BD-diSH. We introduced a cytoplasm targetable dye Calcein-AM (Fig. S5a†) and a nucleus fluorescence marker Hoechst 33342 (Fig. 2p) to confirm the cellular location of BD-diSH in the RAW264.7 cells. As shown in Fig. 2b, f and j, the fluorescence image of BD-diSH exhibited a consistent overlap with Calcein-AM in the cytoplasm, while the probe hardly showed any overlap with the nucleus dye Hoechst 33342 (Fig. 2c, g and k). The color-pair intensity correlation analysis of Fig. 2k was evaluated and shown in Fig. 2m–o and q–s. Only the pixel distribution of BD-diSH and Calcein-AM showed a high correlated plot. The Pearson's colocalization coefficient of BD-diSH and Calcein-AM was $R_r = 0.99$, while the Manders' coefficients were $m_1 = 0.99$ and $m_2 = 0.98$. The results illustrated that BD-diSH could be potentially located in the cytoplasm to detect sulfane sulfur dynamic changes in the cells.

3.6. Imaging of intracellular sulfane sulfur level changes

In order to verify the universal applicability of our as-fabricated probe in the living cells, we next used BD-diSH to image the sulfane sulfur levels in a variety of cell lines. Six types of cell lines were selected to perform the experiments: A549, HEK, HeLa, RH-35, SMMC-7721, and SH-SY5Y. All of the cells were incubated with 1 μM BD-diSH at 37 $^\circ\text{C}$ for 15 min before

imaging. As shown in Fig. 3a (upper row), different levels of weaker fluorescent signals indicated different concentrations of sulfane sulfur in six types of the cell lines. Then, 5 μM Na_2S_4 was added to the above cells for 15 min. The fluorescence intensities in different cell lines were increased (Fig. 3a bottom row). The results demonstrated that the addition of Na_2S_4 was captured by our as-fabricated probe. However, the average fluorescence intensities of the cells shown in Fig. 3a were not identical (Fig. 3b), which revealed that different cell lines might provide different carrying capacities for sulfane sulfur. Flow cytometry analysis was performed to verify the above results. The results (Fig. 3c and d) were consistent with the average fluorescence intensities of the cells observed *via* confocal microscopy in Fig. 3b. All of these results revealed that BD-diSH could respond to the sulfane sulfur level changes in different cell lines.

The intracellular levels of sulfane sulfur have an intimate relationship with the enzymes cystathionine γ -lyase (CSE) and cystathionine β -synthase (CBS).^{6a} We speculated that the interference of the endogenous enzymatic generation systems would disturb the levels of sulfane sulfur in the living cells. We applied the probe BD-diSH to investigate the perturbations of sulfane sulfur biosynthetic pathways. As shown in Fig. 4, the SMMC-7721 cells were divided into four groups. The cells in group **a** (Fig. 4a) were used to image the endogenous level of sulfane sulfur. The weak fluorescent signal indicated that the endogenous concentration of sulfane sulfur was relatively low. The cells in group **b** were induced to overexpress the enzyme CSE (Fig. S9†). The strong increase in fluorescence intensity revealed a high level of sulfane sulfur in these cells. However, if the CSE-overexpressed cells in group **c** were treated with hydroxylamine (HA) to inhibit the CSE activity,¹⁴ the resultant weak fluorescent image suggested a decreased level of sulfane sulfur in these cells. BD-diSH was next applied to image CBS enzyme-mediated sulfane sulfur biosynthesis. The cells in

Fig. 3 Fluorescent imaging of endogenous and exogenous sulfane sulfur level changes in different types of cells by BD-diSH (1 μM) before and after the addition of 5 μM Na_2S_4 . (a) Confocal microscopy images. (b) Histograms of average fluorescence intensities in (a). (c) Flow cytometry analysis. (d) Histograms of mean fluorescence intensities in (c). The data are shown as mean ($\pm$ s.d.) ($n = 7$). The difference was analyzed by one-way ANOVA. *** $P < 0.001$ vs. negative group.

Fig. 4 (a) Confocal fluorescence images of endogenous sulfane sulfur generation in the living cells exposed to different stimulation agents with BD-diSH. The SMMC-7721 cells were stained by 1 μ M BD-diSH for 15 min before imaging. Group a: control; Group b: CSE-overexpress cells; Group c: CSE-overexpressed cells incubated with 250 μ M HA for 30 min; Group d: cells incubated with 3 mM SAM for 1 h. Scale bar: 10 μ m. (b) Corresponding mean fluorescence intensity in (a). (c) Flow cytometry analysis. (d) Corresponding average fluorescence intensity. The data are shown as mean ($\pm$ s.d.) ($n = 7$). The difference was analyzed by one-way ANOVA. *** $P < 0.001$ vs. Group a.

group **d** were stimulated with *S*-adenosyl-L-methionine (SAM) to enhance the activity of CBS.¹⁵ These cells exhibited higher fluorescent signal than that exhibited by group **a**. Fig. 4b displays the mean fluorescence intensities of the cells imaged in Fig. 4a. The confocal fluorescence images demonstrate that our probe could image the endogenous sulfane sulfur generation in the living cells. Flow cytometry analyses were performed to monitor the fluorescence signal changes, which were induced by endogenous sulfane sulfur level fluctuations (Fig. 4c). The results of the average fluorescence intensities of flow cytometry analysis are shown in Fig. 4d. These results were well-consistent with those obtained from the confocal fluorescence images (Fig. 4b and d). All of the above experiments indicated that the probe BD-diSH could competently provide the fluorescent images for the detection of sulfane sulfur in the living cells.

3.7. Evaluation the sulfane sulfur levels in organs

Encouraged by the successful utilization of our probe in imaging sulfane sulfur in the living cells, we attempted to qualitatively evaluate the concentrations of sulfane sulfur in *ex vivo*-dissected organs of mice. The organs were treated as tissue homogenates (tissue 1 g in 10 mL of saline). BD-diSH (10 μ M) was added for 15 min before testing. After the calculations using the regression equation obtained for the curve in Fig. 1c, we obtained the concentrations of sulfane sulfur as 61.3 nmol g^{-1} in the brain, 68.0 nmol g^{-1} in the heart, 64.6 nmol g^{-1} in the liver, 62.3 nmol g^{-1} in the spleen, 25.7 nmol g^{-1} in the lung, and 139 nmol g^{-1} in the kidney, respectively (Fig. 5). Our test results were basically consistent with the reported data.¹⁶ These results indicated that the probe BD-diSH could be applied to assess the concentrations of sulfane sulfur in the isolated organs.

Fig. 5 Qualitative evaluations of sulfane sulfur concentrations in the *ex vivo*-dissected organs. (a) Brain, (b) heart, (c) liver, (d) spleen, (e) lung, and (f) kidney. The fluorescence intensities are presented as normalized. $\lambda_{ex} = 710$ nm and $\lambda_{em} = 736$ nm. The data are shown as mean ($\pm$ s.d.) ($n = 7$).

3.8. Bioimaging sulfane sulfur in mice hippocampus

Hippocampus is a region of the brain that is important for learning and memory.¹⁷ Sulfane sulfur plays a crucial role in the hippocampus functions. We further investigated the level of sulfane sulfur in mice hippocampus with the utility of BD-diSH. The thickness of the hippocampus tissue was 300 μ m. The fluorescent images were obtained from a slice of mice hippocampal tissue incubated with 5 μ g mL^{-1} DAPI (a commercial dye for nucleus) for 30 min and then treated with 20 μ M BD-diSH for 20 min at 37 $^{\circ}C$. The tissue showed weak fluorescence (Fig. 6a), which was caused by the endogenous level of sulfane sulfur in the mice hippocampus. The addition of 20 μ M Na_2S_4

Fig. 6 Fluorescence images of the slices of mice hippocampus. (a) with 20 μM BD-diSH for 20 min. (b) With 5 $\mu\text{g mL}^{-1}$ DIPA for 30 min. (c) Overlay image of (a) and (b). (d) Fluorescence imaging of fresh hippocampus with 20 μM Na_2S_4 for 20 min. (e) Fluorescence imaging of DIPA after the addition of Na_2S_4 . (f) Overlay image of (d) and (e). (g) Three-dimensional (3D) image of (d) accumulated along the z-direction at the depths of 20–105 μm with a magnification of $\times 10$. The representative images were captured using confocal microscopy with Z-stack imaging at the 8.5 μm interval. (h) A 3D image of (e). (i) 3D image of (f). (j) Pseudo-color image of (g) according to fluorescence intensity profiles. (k) Overlay images of (j) and (i). (l) 3D bright-field image of (f). BD-diSH and DAPI images displayed fluorescence intensities collected in emission windows from 700 nm to 800 nm and 425 nm to 500 nm with excitation at 635 nm and 405 nm, respectively.

Fig. 7 *In vivo* imaging of sulfane sulfur level changes in the peritoneal cavity of BALB/c mice. (a) Mice were injected in the i.p. cavity with BD-diSH (50 μ M, 50 μ L in 1 : 9 DMSO/saline, v/v). (b) Mice were injected in the i.p. cavity with Na_2S_4 (50 μ M, 50 μ L) and then injected with BD-diSH (50 μ M, 50 μ L in 1 : 9 DMSO/saline, v/v) for 30 min. (c) Average values of (a) and (b). Quantification of total photon flux from each group. The total number of photons from the entire peritoneal cavity of the mice was integrated. Images were taken after incubation for 30 min. $\lambda_{\text{ex}} = 710$ nm. The data are shown as mean ($\pm$ s.d.) ($n = 7$). The difference was analyzed by one-way ANOVA. *** $P < 0.001$ vs. Group a.

triggered the fluorescence intensity of the mice hippocampus (Fig. 6d), which indicated that our probe could respond to the level fluctuations of sulfane sulfur in the tissue. Fig. 6b and e represent the fluorescent images of the nucleus of mice hippocampus. The overlay images of BD-diSH and DAPI are shown in Fig. 6c and f. To verify the deep tissue imaging capability of the probe, we imaged 20–105 μ m deep axial planes with 8.5 μ m as an interval to construct the three-dimensional (3D) images. The 3D images of Fig. 6d–f along the z-direction with the depths of 20–105 μ m were illustrated in Fig. 6–i. A Z-stack image of the hippocampus showed the activation of fluorescence signal even at a depth of 85 μ m, indicating that our as-fabricated probe has a deep tissue penetrating ability (Fig. 6g). A pseudo-color image of Fig. 6g is displayed in Fig. 6j. The 3D bright-field image of Fig. 6g is shown in Fig. 6l and the overlay image of Fig. 6j–l is shown in Fig. 6k. All of these results demonstrated that BD-diSH had good tissue penetration ability and was capable of detecting sulfane sulfur in the living tissues.

3.9. Bioimaging sulfane sulfur level changes *in vivo*

The excitation and emission spectra in the NIR region can avoid the interferences from the biological samples, penetrate deeply into the tissues, and minimize the photodamage to the biological samples. We next investigated the applicability of BD-diSH for imaging sulfane sulfur *in vivo* by the *in vivo* imaging system. BALB/c mice were divided into two groups (Fig. 7). The mice in group **a** were chosen as the control. The mice in group **b** were administered an intraperitoneal (i.p.) injection of Na_2S_4 (50 μ M, 50 μ L in 1 : 9 DMSO/saline, v/v). These two groups were then administered an i.p. injection of BD-diSH (50 μ M, 50 μ L in 1 : 9 DMSO/saline, v/v) for 30 min.

The mice in group **a** displayed a weak fluorescence signal (Fig. 7a) and the mice in group **b** exhibited stronger fluorescence intensity (Fig. 7b). The mean fluorescence intensities in group **a** and group **b** are shown in Fig. 7c. These results directly indicated that BD-diSH was capable of imaging the Na_2S_4 level changes *in vivo*.

4. Conclusions

In conclusion, we developed a NIR fluorescent probe BD-diSH for the sensitive and selective detection of sulfane sulfur in living cells and *in vivo*. The probe is composed of two moieties: the fluorophore and the response unit, *i.e.*, integrating the response unit 2-mercapto benzoate into the NIR BODIPY fluorophore. The probe can rapidly detect sulfane sulfur within 2 min. This fluorescent probe shows high selective turn-on fluorescence response to sulfane sulfur over bio-relevant RSS, biological reductive materials, as well as various metal ions and anions. BD-diSH can localize at cytoplasm and track the dynamic level changes of sulfane sulfur in the cells. The results indicate that different cell lines contain different levels of sulfane sulfur. Different organs also have different concentrations of sulfane sulfur. The bioimaging assays in the hippocampus and peritoneal cavity of the BALB/c mice illustrate the application of NIR imaging in deep tissue. The results may reveal important information for the inspection of the physiological and pathological roles of sulfane sulfur. Therefore, our as-fabricated BD-diSH probe could potentially provide a powerful new chemical tool for the specific detection and imaging sulfane sulfur in the living cells and *in vivo*.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgements

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Notes and references

- (a) N. Ning, J. Zhu, Y. Du, X. Gao, C. Liu and J. Li, *Nat. Commun.*, 2014, **5**, 4107; (b) B. D. Paul and S. H. Snyder, *Nat. Rev. Mol. Cell Biol.*, 2012, **13**, 499; (c) M. M. Cortese-Krott, A. Koning, G. G. C. Kuhnle, P. Nagy, C. L. Bianco, A. Pasch, D. A. Wink, J. M. Fukuto, A. A. Jackson, H. van Goor, K. R. Olson and M. Feelisch, *Antioxid. Redox Signaling*, 2017, **27**, 684; (d) C. M. Park, L. Weerasinghe, J. J. Day, J. M. Fukuto and M. Xian, *Mol. Biosyst.*, 2015, **11**, 1775.
- (a) M. Iciek and L. Wlodek, *Pol. J. Pharmacol.*, 2001, **53**, 215; (b) J. I. Toohey and A. J. L. Cooper, *Molecules*, 2014, **19**, 12789; (c) E. Cuevasanta, M. N. Möller and B. Alvarez, *Arch. Biochem. Biophys.*, 2017, **617**, 9.
- (a) L. Li, P. Rose and P. K. Moore, *Annu. Rev. Pharmacol.*, 2011, **51**, 169; (b) H. Kimura, *Antioxid. Redox Signaling*, 2010, **12**, 1111; (c) Q. Li and J. R. Lancaster Jr., *Nitric Oxide*, 2013, **35**, 21; (d) G. K. Kolluru, X. Shen, S. C. Bir and C. G. Kevil, *Nitric Oxide*, 2013, **35**, 5.
- V. S. Lin, W. Chen, M. Xian and C. J. Chang, *Chem. Soc. Rev.*, 2015, **44**, 4596.
- (a) K. Ono, T. Akaike, T. Sawa, Y. Kumagai, D. A. Wink, D. J. Tantillo, A. J. Hobbs, P. Nagy, M. Xian, J. Lin and J. M. Fukuto, *Free Radicals Biol. Med.*, 2014, **77**, 82; (b) T. Ida, T. Sawa, H. Ihara, Y. Tsuchiya, Y. Watanabe, Y. Kumagai, M. Suematsu, H. Motohashi, S. Fujii, T. Matsunaga, M. Yamamoto, K. Ono, N. O. Devarie-Baez, M. Xian, J. M. Fukuto and T. Akaike, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 7606.
- (a) J. L. Wood, *Methods Enzymol.*, 1987, **143**, 25; (b) M. Gao, F. Yu, H. Chen and L. Chen, *Anal. Chem.*, 2015, **87**, 3631.
- (a) X. Han, F. Yu, X. Song and L. Chen, *Chem. Sci.*, 2016, **7**, 5098; (b) A. Shibata, Y. Ishima, M. Ikeda, H. Sato, T. Imafuku, V. T. Chuang, Y. Ouchi, T. Abe, H. Watanabe, T. Ishida, M. Otagiri and T. Maruyama, *Biochem. Biophys. Res. Commun.*, 2016, **479**, 578; (c) Y. Huang, F. Yu, J. Wang and L. Chen, *Anal. Chem.*, 2016, **88**, 4122.
- Y. Takano, K. Shimamoto and K. Hanaoka, *J. Clin. Biochem. Nutr.*, 2016, **58**, 7.
- (a) Y. Tang, D. Lee, J. Wang, G. Li, J. Yu, W. Lin and J. Yoon, *Chem. Soc. Rev.*, 2015, **44**, 5003; (b) H. Zhu, J. Fan, J. Du and X. Peng, *Acc. Chem. Res.*, 2016, **49**, 2115; (c) X. Li, X. Gao, W. Shi and H. Ma, *Chem. Rev.*, 2014, **114**, 590; (d) A. S. Klymchenko, *Acc. Chem. Res.*, 2017, **50**, 366.
- (a) K. Umezawa, M. Yoshida, M. Kamiya, T. Yamasoba and Y. Urano, *Nat. Chem.*, 2017, **9**, 279; (b) R. S. Kathayat, P. D. Elvira and B. C. Dickinson, *Nat. Chem. Biol.*, 2017, **13**, 150; (c) A. Sargenti, G. Farruggia, N. Zaccheroni, C. Marraccini, M. Sgarzi, C. Cappadone, E. Malucelli, A. Procopio, L. Prodi, M. Lombardo and S. Iotti, *Nat. Protoc.*, 2017, **12**, 461; (d) X. Han, F. Yu, X. Song and L. Chen, *Adv. Funct. Mater.*, 2017, **27**, 1700769; (e) H. Zhang, J. Liu, C. Liu, P. Yu, M. Sun, X. Yan, J. P. Guo and W. Guo, *Biomaterials*, 2017, **133**, 60; (f) X. Han, F. Yu, X. Song and L. Chen, *Chem. Sci.*, 2017, **8**, 6991; (g) Y. Hu, L. Shi, Y. Su, C. Zhang, X. Jin and X. Zhu, *Biomater. Sci.*, 2017, **5**, 792.
- (a) W. Chen, C. Liu, B. Peng, Y. Zhao, A. Pacheco and M. Xian, *Chem. Sci.*, 2013, **4**, 2892; (b) C. Liu, W. Chen, W. Shi, B. Peng, Y. Zhao, H. Ma and M. Xian, *J. Am. Chem. Soc.*, 2014, **136**, 7257; (c) W. Chen, E. W. Rosser, T. Matsunaga, A. Pacheco, T. Akaike and M. Xian, *Angew. Chem., Int. Ed.*, 2015, **54**, 13961; (d) W. Chen, A. Pacheco, Y. Takano, J. J. Day, K. Hanaoka and M. Xian, *Angew. Chem., Int. Ed.*, 2016, **55**, 9993; (e) W. Chen, A. Pacheco, Y. Takano, J. J. Day, K. Hanaoka and M. Xian, *Org. Lett.*, 2015, **17**, 2776.
- (a) L. Zeng, S. Chen, T. Xia, W. Hu, C. Li and Z. Liu, *Anal. Chem.*, 2015, **87**, 3004; (b) H. Shang, H. Chen, Y. Tang, R. Guo and W. Lin, *Sens. Actuators, B*, 2016, **230**, 773; (c) Y. Takano, K. Hanaoka, K. Shimamoto, R. Miyamoto, T. Komatsu, T. Ueno, T. Terai, H. Kimura, T. Nagano and Y. Urano, *Chem. Commun.*, 2017, **53**, 1064; (d) M. Ikeda, Y. Ishima, A. Shibata, V. T. G. Chuang, T. Sawa, H. Ihara, H. Watanabe, M. Xian, Y. Ouchi, T. Shimizu, H. Ando, M. Ukawa, T. Ishida, T. Akaike, M. Otagiri and T. Maruyama, *Anal. Chim. Acta*, 2017, **969**, 18; (e) Y. Takano, H. Echizen and K. Hanaoka, *Antioxid. Redox Signaling*, 2017, **27**, 669; (f) Q. Han, Z. Mou, H. Wang, X. Tang, Z. Dong, L. Wang, X. Dong and W. Liu, *Anal. Chem.*, 2016, **88**, 7206; (g) J. Ma, J. Fan, H. Li, Q. Yao, F. Xu, J. Wang and X. Peng, *J. Mater. Chem. B*, 2017, **5**, 2574.
- (a) F. Yu, M. Gao, M. Li and L. Chen, *Biomaterials*, 2015, **63**, 93; (b) J. Li, Z. Zhu, S. Rong, H. Li, Y. Guo, Q. Xue and D. Ding, *Biomater. Sci.*, 2017, **5**, 1622; (c) L. Yuan, W. Lin, K. Zheng, L. He and W. Huang, *Chem. Soc. Rev.*, 2013, **42**, 622; (d) X. Jing, F. Yu and L. Chen, *Chem. Commun.*, 2014, **50**, 14253; (e) P. Liu, X. Jing, F. Yu, C. Lv and L. Chen, *Analyst*, 2015, **140**, 4576; (f) F. Yu, P. Li, B. Wang and K. Han, *J. Am. Chem. Soc.*, 2013, **135**, 7674.
- A. Asimakopoulou, P. Panopoulos, C. T. Chasapis, C. Coletta, Z. Zhou, G. Cirino, A. Giannis, C. Szabo,

- G. A. Spyroulias and A. Papapetropoulos, *Br. J. Pharmacol.*, 2013, **169**, 922.
- 15 (a) T. Ubuka, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2002, **781**, 227; (b) C. Liu, F. Zhang, G. Munske, H. Zhang and M. Xian, *Free Radicals Biol. Med.*, 2014, **76**, 200.
- 16 K. Módis, C. Coletta, A. Asimakopoulou, B. Szczesny, C. Chao, A. Papapetropoulos, M. R. Hellmich and C. Szabo, *Nitric Oxide*, 2014, **41**, 146.
- 17 P. Rivera-Fuentes, A. T. Wrobel, M. L. Zastrow, M. Khan, J. Georgiou, T. T. Luyben, J. C. Roder, K. Okamoto and S. J. Lippard, *Chem. Sci.*, 2015, **6**, 1944.