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A Fluorescent Probe for Selective Facile Detection of H_2S in Serum Based on an Albumin-Binding Fluorophore and Effective Masking Reagent

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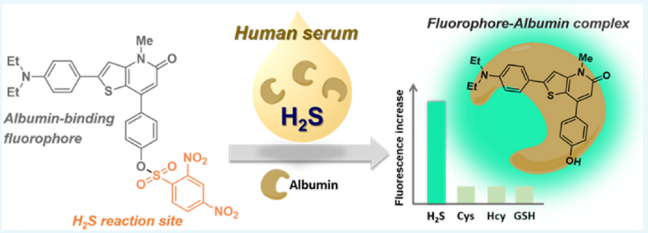
ABSTRACT: A fluorescent probe (4-(2-(4-(diethylamino)-phenyl)-4-methyl-5-oxo-4,5-dihydrothieno[3,2-*b*]pyridin-7-yl)-phenyl 2,4-dinitrobenzenesulfonate, **KF-DNBS**) for facile detection of H_2S in serum was developed based on the combination of an environment-sensitive fluorophore (2-(4-(diethylamino)-phenyl)-7-(4-hydroxyphenyl)-4-methylthieno[3,2-*b*]pyridin-5(4*H*)-one, **KF**) with albumin and the 2,4-dinitrobenzene sulfonyl (DNBS) group as a recognition unit for H_2S. **KF-DNBS** showed remarkable fluorescence enhancement due to H_2S-triggered thiolysis followed by the formation of a fluorescent fluorophore (**KF**)-albumin complex. The H_2S detection limit of **KF-DNBS** was estimated to be 3.2 μM, and **KF-DNBS** achieves a high selectivity to H_2S over biothiols by employing 2-formyl benzene boronic acid (2-FBBA) as an effective masking reagent. Furthermore, under optimized sensing conditions, **KF-DNBS** could be applied to accurately determine spiked H_2S in human serum without the need for any further procedure for the removal of serum proteins.



INTRODUCTION

Recently, hydrogen sulfide (H_2S) has emerged as a significant endogenous gaseous mediator, like the already well-known nitric oxide (NO) and carbon monoxide (CO).[1−3] H_2S is produced through enzymatic reactions of cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST)/cysteine aminotransferase (CAT) in human tissue, and it is widely distributed in the human body in concentrations ranging from 10 to 100 μM.[4] H_2S has a variety of physiological functions, such as anti-inflammation, neuromodulation, and vasoregulation.[5−9] Due to its clinical implications, the perturbed synthesis of endogenous H_2S is closely associated with various human diseases. Recent studies have shown that abnormal serum levels of H_2S are observed in several physiological disorders, such as Alzheimer's disease, hypertension, diabetes, and asthma.[2,10−14] Hence, the development of a reliable detection method for H_2S in serum has great importance in pathology. Moreover, fast and real-time monitoring is desirable considering the rapid metabolism of H_2S in physiological processes.

To date, a variety of analytical techniques such as spectrophotometry, electrochemical assay, and chromatography (including gas, ion-exchange, and variants of high-performance liquid chromatography (HPLC)) have been reported for H_2S detection.[15−19] Among them, two common methods have been widely used for measuring H_2S levels in serum: a colorimetric method using methylene blue (MB method) and an ion-selective electrode (ISE)-based sulfide anion (S^{2-})-specific method.[20−22] Both methods are performed under harsh chemical conditions that may lead to overestimation, and they also possess several practical drawbacks, such as tedious sample processing and the requirement of sophisticated instruments.

In contrast, fluorescent small-molecule probes have great potential for real-time monitoring of H_2S in terms of their simplicity, rapid response, and high sensitivity.[23−26] In past decades, various reaction-based H_2S fluorescent probes have been developed based on the intrinsic reactivity of H_2S as a good reducing agent or a good nucleophile. The majority of them employed photoinduced electron transfer (PET) modulated by an electron-withdrawing azide or 2,4-dinitrobenzyl group, which are commonly used in H_2S probes.[27−33] A H_2S-triggered reaction releases the fluorophore, and this is followed by a turn-on fluorescence response. Such fluorophores have shown successful performance in the detection of H_2S at low levels. However, most of them are focused on the fluorescence imaging of H_2S, and it is intricate to be applied for the measurement of H_2S levels in serum samples since they suffer signal interference due to nonspecific binding of the fluorophore with serum proteins (Scheme 1a). This problem is found in most prevalent fluorophores, such as coumarin,

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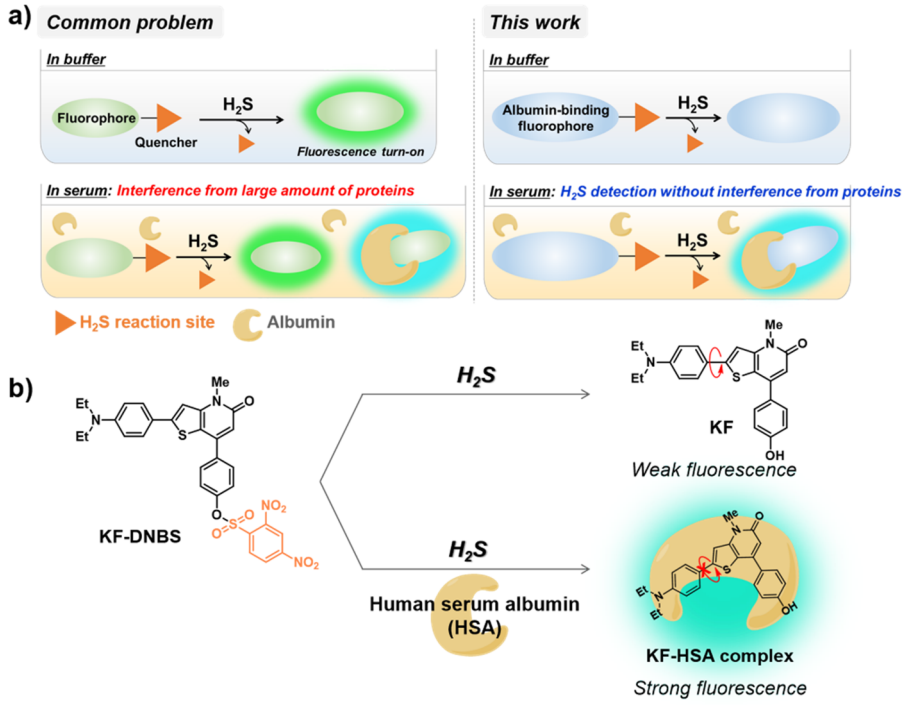
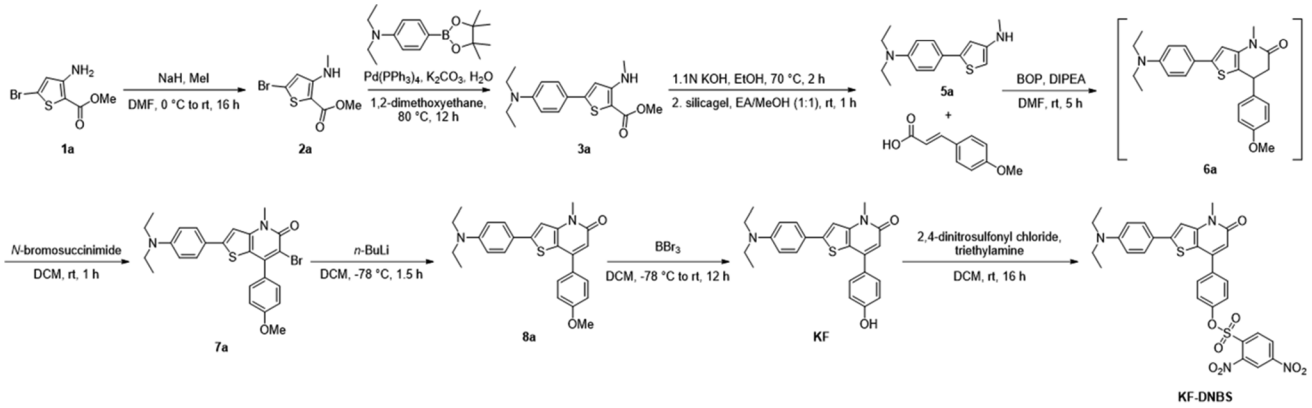
Scheme 1. (a) Summary of Common Problems in Application of Conventional Fluorescent Probes for H_2S in Serum. (b) Schematic Illustration of Application of the Fluorescent KF-DNBS Probe for Facile H_2S Detection in Serum Using H_2S-Triggered Cascade Formation of the Fluorescent KF-Albumin Complex



Scheme 2. Synthetic Route for KF-DNBS



BODIPY, rosamine, and so on.[34−37] To avoid signal interference, which could lead to inaccurate measurement of H_2S in serum, an additional process to remove the large amounts of proteins in serum samples before H_2S measurement is essential. In addition, many thiolysis-based probes are susceptible to interference from other biothiols present in high concentrations in serum, such as cysteine (Cys) and homocysteine (Hcy), which have similar reactivity to H_2S. Thus, it is still necessary to develop a highly selective fluorescent probe that is readily applicable to H_2S quantitation in serum samples.

Recently, we have reported a new sensing strategy to detect enzyme activity in human serum using a fluorescent probe.[38] A caged fluorophore was designed as a probe by conjugation of an enzyme-selective recognition unit with an environment-sensitive fluorophore. The fluorescence of the latter strongly depends on the specific binding of the fluorophore to human serum albumin (HSA), which is present in serum at a very high concentration. The caged fluorophore enabled quantitative detection of the serum enzyme without any pretreatment. In this study, we utilized the strategy to develop a fluorescent chemodosimeter for H_2S detection without interference from serum proteins and prepared a caged fluorophore (**KF-DNBS**) by conjugating an environment-sensitive thienopyridinone derivative (**KF**) with the 2,4-dinitrobenzene sulfonyl (**DNBS**) group.[39] The fluorescence of **KF** strongly depends on its specific binding to HSA, and the DNBS group is sensitively cleaved by H_2S. **KF-DNBS** showed weak fluorescence intensity in the absence and presence of albumin. As illustrated in Scheme 1b, in response to H_2S, the DNBS group in **KF-DNBS** was cleaved by thiolysis, thus releasing **KF**, which immediately combined with albumin, resulting in significant fluorescence enhancement. Based on this H_2S-triggered cascade formation of the fluorescent **KF**-albumin complex, **KF-DNBS** could be used in the quantitative detection of H_2S in physiological conditions and could also

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be applied to serum samples without additional processing for the removal of serum proteins.

RESULTS AND DISCUSSION

Design and Synthesis of KF-DNBS. Serum contains various proteins, such as albumin, globulin, transferrin, and fibrinogen.[40] Among them, albumin is present at the highest concentration of 600 μM or more. In the presence of other serum proteins, **KF**, which is an environment-sensitive fluorophore derived from thienopyridinone, originally exhibited weak fluorescence, but this increased immediately when 2 equiv. of HSA was added, as shown in Figure S1. In addition, a binding constant (K_a) of **KF** for HSA based on the Benesi–Hildebrand plot was determined to be $K_a = 1.9 \times 10^4$ M^{-1} showing strong binding between **KF** and HSA (Figure S2).[41] By utilizing the albumin-specific fluorescence turn-on response of **KF**, we designed caged **KF** as a fluorescent probe for facile H_2S detection in serum and synthesized the **KF-DNBS** probe through modification of the phenolic site of **KF** with a well-known H_2S recognition unit, namely, the DNBS group (Scheme 2).

Then, we compared the fluorescence responses of **KF** and **KF-DNBS** to HSA. As shown in Figure 1, the fluorescence

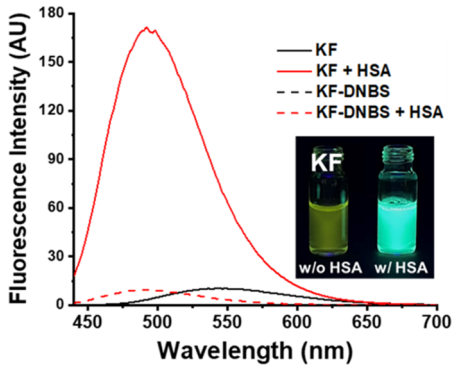

Figure 1. Fluorescence spectra of **KF** and **KF-DNBS** (each 25 μM, 10% DMSO) in the absence and presence of HSA (100 μM), λ_{ex} = 420 nm; fluorescence image of **KF** in the absence and presence of HSA under handheld UV lamp (365 nm) illumination (inset).

intensity of **KF** shifted slightly from 550 to 500 nm by addition of HSA and increased linearly with increasing HSA concentration in the range of 5–50 μM (Figure S3). In contrast to **KF**, **KF-DNBS** showed weak fluorescence with or without excess HSA. Also, the fluorescence of **KF** and **KF-DNBS** remained constant before and after irradiation or heating showing excellent photo- and thermostability (Figure S4). Based on these results, we envisioned that **KF-DNBS** with HSA could be utilized as a reaction-based fluorescent probe for H_2S using the principle of H_2S-triggered cascade formation of the fluorescent **KF**-HSA complex (Φ = 0.546).

Optimization of Sensing Conditions for Selective H_2S Detection. The 2,4-dinitrosulfonyl unit including DNBS has been the most frequently used H_2S recognition unit in H_2S-reactive fluorescent probes. However, these probes usually possessed moderate selectivity because of interference from other biothiols, such as Cys, Hcy, and GSH. Thus, it is desirable to establish the optimal sensing conditions for highly selective H_2S detection by **KF-DNBS**, especially for reliable application in serum samples containing high concentrations of Cys and Hcy. First, we evaluated the relative reactivity of H_2S and other biothiols, including Cys, Hcy, and GSH, to **KF-DNBS** considering their approximate concentrations in human serum ([H_2S] = 100 μM, [Cys] = 250 μM, [Hcy] = 100 μM, [GSH] = 10 μM).[42] As shown in Figure 2a, when H_2S was

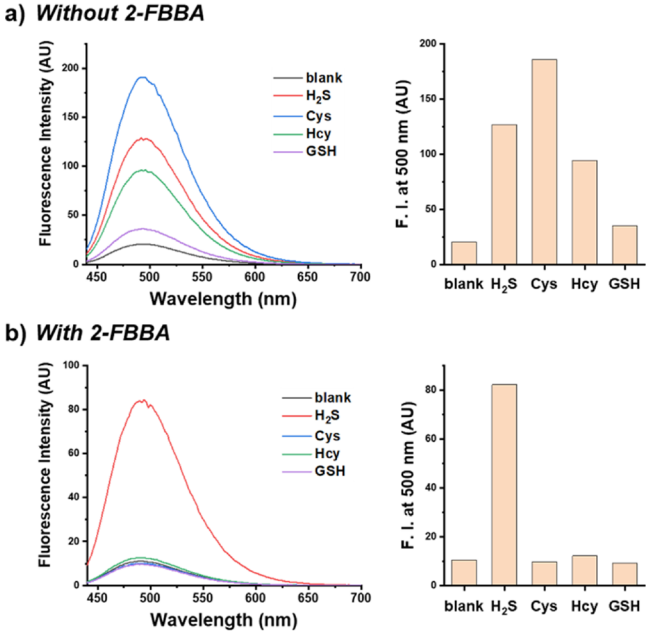

Figure 2. Change of fluorescence intensity of **KF-DNBS** (25 μM, 10% DMSO) with HSA (100 μM) upon addition of each biothiol, H_2S (100 μM), Cys (250 μM), Hcy (100 μM), GSH (10 μM) in SPB (pH 7.4, 20 mM), (a) without 2-FBBA and (b) with 2-FBBA (2 mM), λ_{ex} = 420 nm, 37 °C.

added to the sample solution containing **KF-DNBS** with HSA in SPB (pH 7.4), the fluorescence intensity at 500 nm increased significantly as we expected. However, Cys and Hcy also induced a noticeable fluorescence change.

To solve this problem, we tried to introduce a masking reagent, which can block the nucleophilic reactivity of Cys and Hcy selectively by the formation of a stable covalent bond. Since the cyclization reaction between aldehyde groups and Cys or Hcy has been widely used in selective probe molecule design, we investigated simple aldehydes, and 2-formyl benzene boronic acid (2-FBBA) was chosen as a potential masking reagent. 2-FBBA is a well-known reagent used in facile and selective bioconjugation of N-terminal Cys in proteins at neutral pH.[43,44] It enables very rapid formation of a stable thiazolidino boronate complex with the boronic acid moiety by means of a B–N dative bond. We envisioned that the introduction of 2-FBBA would improve the selectivity of **KF-DNBS** to H_2S by blocking the reactivity of Cys and Hcy based on the fast and chemoselective reaction of 2-FBBA with Cys and Hcy.

We confirmed the kinetics and selectivity of the reaction between 2-FBBA and biothiols by monitoring the intrinsic absorption of 2-FBBA, which decreased as the aldehyde in 2-FBBA converted to thiazolidine.[43] As shown in Figure S6, the rapid (<100 s) decrease of absorbance at 254 nm was observed only with Cys and Hcy, indicating a rapid and selective reaction with Cys and Hcy. Next, the capability of 2-FBBA as a masking reagent for selective H_2S detection using **KF-DNBS** was investigated. **KF-DNBS** with HSA in the presence of 2-FBBA showed superior selectivity to H_2S over other biothiols

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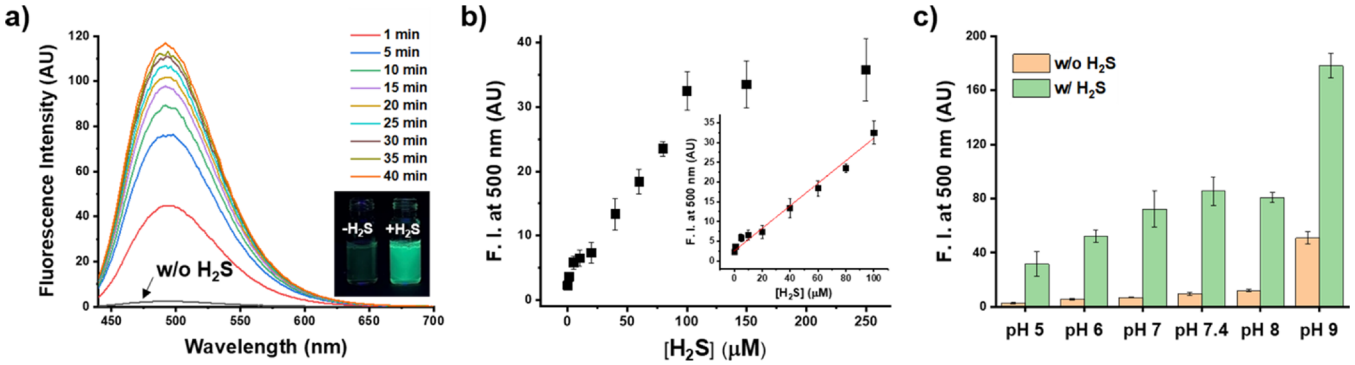

Figure 3. (a) Fluorescence spectral change of **KF-DNBS** (25 μM, 10% DMSO) with HSA (100 μM) in the absence and presence of H_2S (100 μM) in SPB (pH 7.4, 20 mM) containing 2-FBBA (2 mM); the corresponding fluorescence image taken under handheld UV lamp (365 nm) illumination after 5 min (inset). (b) Plot of fluorescence intensity at 500 nm of **KF-DNBS** (25 μM, 10% DMSO) with HSA (100 μM) versus different concentrations of H_2S (5−250 μM) in SPB (pH 7.4, 20 mM) containing 2-FBBA (2 mM). (c) Fluorescence intensity at 500 nm of **KF-DNBS** (25 μM, 10% DMSO) with HSA (100 μM) in the absence and presence of H_2S (100 μM) in various buffer conditions (pH 5−9, 20 mM), λ_{ex} = 420 nm, 37 °C.

(Figure 2b). 2-FBBA was able to completely block the reactivity of Cys and Hcy upon **KF-DNBS**. These results showed, for the first time, that 2-FBBA could be used as an effective masking reagent for Cys and Hcy in thiolysis-based H_2S probes. We investigated further by using **KF-DNBS** as a H_2S probe under the following conditions: 25 μM **KF-DNBS**, 100 μM HSA, and 2-FBBA (2 mM) in SPB (pH 7.4, 20 mM).

Sensing Behavior of KF-DNBS with HSA as a Selective H_2S Probe. The feasibility of **KF-DNBS** with HSA as a H_2S probe was investigated under the optimized measurement conditions. First, we examined the spectral change of **KF-DNBS** with HSA in response to H_2S. When H_2S was added to the sensing system containing **KF-DNBS** with HSA and the masking reagent 2-FBBA in SPB, the intrinsic absorption peak of the probe (λ_{abs} = 420 nm) gradually increased and significant enhancement of fluorescence intensity at 500 nm appeared immediately (Figure S7, Figure 3a). The intensity reached the maximum value within 20 min. As shown in Figure 3b, the fluorescence intensity of **KF-DNBS** with HSA increased linearly with increasing H_2S concentration (5−100 μM). The detection limit (3σ/slope) was determined to be 3.2 μM, which is reliable for the measurement of serum H_2S levels. In addition, the fluorescence change of the sample solution could be monitored by the naked eye, and it was confirmed that **KF-DNBS** with HSA worked well in a broad pH range from pH 5 to pH 9 (Figure 3c).

To further confirm that the turn-on fluorescence was due to the H_2S-triggered cascade formation of the fluorescent **KF**-HSA complex, we confirmed the product of the thiolysis reaction of **KF-DNBS** by HPLC and ^{1}H NMR studies. In the HPLC chromatogram, shown in Figure S8, a peak with a retention time consistent with that of **KF** appeared when **KF-DNBS** was incubated with H_2S in SPB solution and gradually increased with increasing H_2S concentration. Additionally, the ^{1}H NMR spectrum of the sample solution containing **KF-DNBS** with H_2S showed that the peaks of the 2,4-dinitrophenyl by-product (δ 8.95, 8.45) increased (Figure S9). Based on these results, we established that the fluorescence response of **KF-DNBS** with HSA to H_2S was caused by thiolysis-induced **KF** release followed by the formation of a fluorescent **KF**-HSA complex.

Next, the selectivity of **KF-DNBS** with HSA toward H_2S was investigated using various biologically relevant species, including biothiols (Cys, Hcy, GSH), reactive sulfur species (RSS) and reactive oxygen species (ROS) (HSO_4^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, SCN^-, H_2O_2, ClO^-), and anions (CN^-, F^-, Br^-, NO_3^-, NO_2^-, HCO_3^-, $CH_3CO_2^-$). As shown in Figure 4,

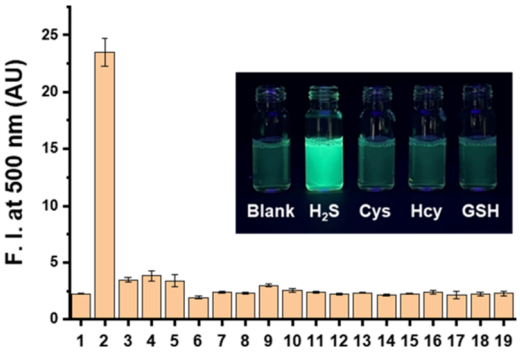

Figure 4. Fluorescence intensity at 500 nm of **KF-DNBS** (25 μM, 10% DMSO) with HSA (100 μM) in the presence of various analytes: 1: blank, 2: H_2S (100 μM), 3: Cys (250 μM), 4: Hcy (100 μM), 5: GSH (10 μM), 6: HSO_4^- (100 μM), 7: SO_4^{2-} (100 μM), 8: SO_3^{2-} (100 μM), 9: $S_2O_3^{2-}$ (100 μM), 10: SCN^- (100 μM), 11: CN^- (100 μM), 12: F^- (100 μM), 13: Br^- (100 μM), 14: NO_3^- (100 μM), 15: NO_2^- (100 μM), 16: HCO_3^- (100 μM), 17: $CH_3CO_2^-$ (100 μM), 18: H_2O_2 (100 μM), 19: ClO^- (100 μM) in SPB (pH 7.4, 20 mM), λ_{ex} = 420 nm, 37 °C; the corresponding fluorescence image after 5 min under handheld UV lamp (365 nm) illumination (inset).

the remarkable fluorescence enhancement was observed only for H_2S. The specific response to H_2S suffered no interference by other analytes (Figure S10), and we could confirm the selectivity for H_2S over other biothiols with the naked eye. This result demonstrated that **KF-DNBS** with HSA and the masking reagent 2-FBBA exhibits a high selectivity toward H_2S and would work well in complex serum samples containing many biothiols and other reactive species.

Application of KF-DNBS in Quantitative Detection of H_2S in Human Serum. To explore the potential applicability of **KF-DNBS** to facile detection of H_2S in serum, the fluorescence response of **KF-DNBS** to H_2S-spiked human serum samples was investigated. Human serum (purchased from Sigma Aldrich) was spiked with different concentrations of H_2S (25, 50, 100, 150 μM). The serum samples, without any pretreatment, were directly added to a solution containing 2-

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FBBA in SPB. Since it has been reported that the concentration of HSA in human serum ranges from 550 to 800 μM, there was no need to add HSA to the sample solutions. After the addition of **KF-DNBS**, the change in fluorescence intensity of the sample solution was measured. Based on the calibration curve obtained from the plot of the initial rate of fluorescence change for 10 min versus the concentration of H_2S using **KF-DNBS** with HSA (Figure S11), we could determine the spiked H_2S level in HSA and the recovery ranged from 95 to 109%, as summarized in Table 1.

Table 1. Determination of Spiked H_2S in Human Serum Using KF-DNBS

	added (μM)	found (μM)	recovery (%)	RSD[a] (%)
human serum	25	26	106	13
	50	51	101	10
	100	95	95	11
	150	163	109	12

[a]Relative standard deviation.

This result showed that the fluorescence response of **KF-DNBS** to spiked H_2S was unaffected by the various analytes present in human serum, including high concentrations of biothiols as well as proteins, even though no additional process was performed prior to sample measurement. Thus, the fluorescence response of **KF-DNBS** could be utilized as an accurate and facile method for measuring H_2S levels in serum samples.

CONCLUSIONS

We developed a highly selective fluorescent probe **KF-DNBS** for H_2S detection based on a H_2S-triggered thiolysis reaction. Upon addition of H_2S, **KF-DNBS** with HSA showed rapid (within 5 min) and remarkable fluorescence enhancement and responded to very low concentrations of H_2S (LOD = 3.2 μM). **KF-DNBS** exhibited high selectivity toward H_2S, especially over other biothiols including Cys, Hcy, and GSH, by employing 2-FBBA as an effective masking reagent for Cys and Hcy. 2-FBBA completely blocked the reactivity of Cys and Hcy by a fast and chemoselective reaction. Moreover, **KF-DNBS** could be applied for the simple, quantitative analysis of spiked H_2S in human serum, without the need for any additional protein removal procedure. Consequently, this study presents a facile method to detect H_2S in serum samples in various H_2S-related diseases. We also demonstrated, for the first time, the application of 2-FBBA as a Cys/Hcy-masking reagent in H_2S probes, as a valuable approach to improve the selectivity of many thiolysis-based H_2S sensing systems.

EXPERIMENTAL SECTION

Materials and Instruments. All the biological analytes and other chemical reagents were purchased from commercial suppliers (Sigma Aldrich, Tokyo Chemical Industry, Alfa Aesar, and UChem) and used without further purification. All melting points were recorded on a micromelting point apparatus and are stated uncorrected. Reactions were monitored by thin layer chromatography (TLC) with 0.25 mm precoated silica gel plates (Kieselgel $60F_{254}$). Flash column chromatography was performed on silica gel (70−230 mesh) using distilled organic solvents. Fluorescence spectra were recorded using a Cytation 3 Multi-Mode Reader and an Agilent Cary Eclipse fluorescence spectrophotometer. All UV−vis spectra were recorded using an Agilent Cary 8454 UV−vis spectrophotometer. ^{1}H NMR and $^{13}C\{^1H\}$ NMR spectra were obtained using a Bruker Advance III HD 600 MHz spectrometer. Chemical shifts are reported as δ (ppm) values relative to chloroform ($CDCl_3$, δ 7.260) and dimethyl sulfoxide (DMSO-d_6, δ 2.50), and coupling constants are reported in Hz. High-resolution mass spectroscopy (HRMS) data were obtained using a magnetic sector-electric sector double focusing mass analyzer at the Korea Basic Science Institute (KBSI) in electron spray ionization (ESI) mode and measured by time of flight (TOF) in ESI mode on an SCIEX X500R QTOF.

Synthesis of the Probe. The synthetic route of probe **KF-DNBS** is shown in Scheme 2. The key intermediate compound **5a** was synthesized according to the modified method from the previously reported methods.[45]

*Synthesis of Methyl 5-(4-(Diethylamino)phenyl)-3-(methylamino)thiophene-2-carboxylate (**3a**).* To a solution of **2a** (1.04 g, 4.14 mmol, 1.0 equiv.) in 1,2-dimethoxyethane (13.8 mL, 0.3 M), $Pd(PPh_3)_4$ (239.4 mg, 0.2072 mmol, 0.05 equiv.), *N*,*N*-diethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (1.37 g, 4.97 mmol, 1.2 equiv.), K_2CO_3 (1.72 g, 12.43 mmol, 3.0 equiv.), and H_2O (0.3 mL) were added. The reaction mixture was heated at 80 °C and stirred for 12 h. After completion of the reaction, the mixture was filtered through celite and extracted with H_2O/EtOAc three times. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude was purified by flash column chromatography with *n*-hexane:EtOAc (5:1) on silica to afford **3a** (1.26 g, 3.95 mmol, 96%) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.50 (d, J = 9.0 Hz, 2H), 6.70 (s, 1H), 6.65 (d, J = 9.0 Hz, 2H), 3.81 (s, 3H), 3.33 (q, J = 7.0 Hz, 4H), 3.02 (d, J = 4.8 Hz, 3H), 1.19 (t, J = 7.2 Hz, 6H); $^{13}C\{^1H\}$ NMR (150 MHz, $CDCl_3$) δ 165.3, 158.0, 151.4, 148.3, 127.3, 120.5, 111.4, 108.7, 94.9, 50.9, 44.4, 31.6, 12.6; Data are consistent with those reported in the literature.[45]

*Synthesis of 6-Bromo-2-(4-(diethylamino)phenyl)-7-(4-methoxyphenyl)-4-methylthieno[3,2-b]pyridin-5(4H)-one (**7a**).* To a solution of **5a** (150 mg, 0.5760 mmol, 1.0 equiv.) in DMF (2.3 mL, 0.25 M), 4-methoxycinnamic acid (225.8 mg, 1.267 mmol, 2.2 equiv.), (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP, 560 mg, 1.267 mmol, 2.2 equiv.), and DIPEA (0.51 mL, 2.880 mmol, 5.0 equiv.) were added at room temperature. The mixture was stirred at room temperature for 5 h. The reaction was quenched with H_2O and extracted with ethyl acetate (EtOAc) three times. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude was used without further purification. To a solution of mixture **6a** (188.0 mg, 0.4813 mmol, 1.0 equiv.) in CH_2Cl_2 (4.8 mL, 0.1 M), *N*-bromosuccinimide (128.5 mg, 0.7221 mmol, 1.5 equiv.) was added. The mixture was stirred at room temperature for 1 h. The reaction was quenched with H_2O and extracted with EtOAc three times. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude was purified by flash column chromatography with *n*-hexane:EtOAc (1:1) on silica to afford **7a** as a yellow solid (22.4 mg, 0.0532 mmol, 48%, over two steps); mp: 212−214 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.45−7.41 (m, 4H), 7.05 (s, 1H), 7.03 (d, J = 8.4 Hz, 2H), 6.62 (d, J = 9.0 Hz, 2H), 3.88 (s, 3H), 3.82 (s, 3H), 3.38 (q, J = 7.0 Hz, 4H), 1.18 (t, J = 6.9 Hz, 6H); $^{13}C\{^1H\}$ NMR (150 MHz, $CDCl_3$) δ 160.2, 159.3, 150.8, 148.6, 146.4,

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143.2, 130.2, 129.9, 127.3, 119.9, 118.4, 114.1, 111.6, 111.3, 108.7, 55.4, 44.5, 33.6, 12.7; HRMS (EI): m/z calcd for $C_{25}H_{25}BrN_2NaO_2S$ $[M + Na]^+$ 519.0718, found 519.0719.

*Synthesis of 2-(4-(Diethylamino)phenyl)-7-(4-methoxyphenyl)-4-methylthieno[3,2-b]pyridin-5(4H)-one (**8a**).* A solution of **7a** (36.1 mg, 0.0726 mmol, 1.0 equiv.) in CH_2Cl_2 (0.73 mL, 0.1 M) was cooled down to −78 °C. Next, *n*-butyllithium solution (2.0 M in cyclohexane, 75 μL, 0.109 mmol, 1.5 equiv.) was slowly added and stirred at −78 °C for 1.5 h. The reaction was quenched with H_2O. The mixture was extracted with CH_2Cl_2 three times. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude was purified by flash column chromatography with *n*-hexane:EtOAc (1:1 to 100% EtOAc) on silica to afford **8a** as a yellow solid (15.8 mg, 0.0377 mmol, 52%); mp: 190−192 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.64 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 8.4 Hz, 2H), 7.10 (s, 1H), 7.02 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 6.51 (s, 1H), 3.88 (s, 3H), 3.76 (s, 3H), 3.40 (q, J = 7.2 Hz, 4H), 1.19 (t, J = 6.9 Hz, 6H); ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 163.0, 160.7, 150.1, 148.5, 146.7, 145.3, 130.1, 129.1, 127.4, 120.3, 116.4, 114.5, 113.3, 111.7, 109.2, 55.5, 44.6, 31.9, 12.7; HRMS (EI): m/z calcd for $C_{25}H_{26}N_2O_2S$ $[M]^+$ 418.1715, found 418.1711.

*Synthesis of 2-(4-(Diethylamino)phenyl)-7-(4-hydroxyphenyl)-4-methylthieno[3,2-b]pyridin-5(4H)-one (**KF**).* A solution of **8a** (109.8 mg, 0.2623 mmol, 1.0 equiv.) in CH_2Cl_2 (2.6 mL, 0.1 M) was cooled down to −78 °C. After cooling, boron tribromide in CH_2Cl_2 solution (3.94 mL, 3.935 mmol, 15.0 equiv.) was added slowly, dropwise, to the solution. Next, the mixture was warmed up to room temperature and stirred for 12 h. After the solution was cooled down to −78 °C, the reaction was quenched with iced water and neutralized with $NaHCO_3$. The mixture was extracted with CH_2Cl_2 three times. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude was purified by flash column chromatography with *n*-hexane:EtOAc (1:1 to 100% EtOAc) on silica to afford **KF** as a yellow solid (83.0 mg, 0.2052 mmol, 78%); mp: 250−251 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.94 (s, 1H), 7.60−7.58 (m, 3H), 7.56 (d, J = 9.0 Hz, 2H), 6.93 (d, J = 8.4 Hz, 2H), 6.70 (d, J = 9.0 Hz, 2H), 6.27 (s, 1H), 3.65 (s, 3H), 3.38 (q, J = 7.0 Hz, 4H), 1.11 (t, J = 7.2 Hz, 6H); ^{13}C{^{1}H} NMR (150 MHz, DMSO-d_6) δ 161.4, 158.8, 148.7, 148.0, 145.9, 145.5, 128.8, 127.6, 127.0, 119.2, 115.8, 114.0, 111.8, 111.4, 110.4, 43.7, 31.4, 12.4; HRMS (EI): m/z calcd for $C_{24}H_{24}N_2O_2S$ $[M]^+$ 404.1558, found 404.1555.

*Synthesis of 4-(2-(4-(Diethylamino)phenyl)-4-methyl-5-oxo-4,5-dihydrothieno[3,2-b]pyridin-7-yl)phenyl 2,4-Dinitrobenzenesulfonate (**KF-DNBS**).* To a solution of **KF** (106.5 mg, 0.2633 mmol, 1.0 equiv.) in CH_2Cl_2 (2.6 mL, 0.1 M), 2,4-dinitrobenzenesulfonyl chloride (119.3 mg, 0.4476 mmol, 1.7 equiv.) and triethylamine (0.13 mL, 1.250 mmol, 4.7 equiv.) were added. The mixture was stirred at room temperature for 16 h. The reaction was quenched with H_2O and extracted with CH_2Cl_2 three times. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude was purified by flash column chromatography with *n*-hexane:EtOAc (1:1 to 1:5) on silica to afford **KF-DNBS** as a dark brown solid (108.6 mg, 0.1711 mmol, 65%, conversion: 82%, borsm yield: 79%); mp: 96−98 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.67 (s, H), 8.51 (d, J = 8.4 Hz, 2H), 8.25 (d, J = 8.4 Hz, 2H), 7.67 (d, J = 7.8 Hz, 2H), 7.46 (d, J = 7.2 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 7.09 (s, 1H), 6.65 (br s, 2H), 6.44 (s, 1H), 3.73 (s, 3H), 3.41 (q, J = 7.2 Hz, 4H), 1.12 (t, J = 6.9 Hz, 6H); ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 162.6, 151.1, 150.6, 149.4, 149.1, 148.7, 145.6, 137.7, 134.0, 133.6, 129.7, 127.4, 126.7, 122.7, 120.5, 119.7, 115.4, 114.1, 111.6, 109.1, 44.5, 31.9, 12.6; HRMS (ESI): m/z calcd for $C_{30}H_{27}N_4O_8S_2$ $[M + H]^+$ 635.1270, found 635.1262.

Comparison of the Fluorescence Change of KF and KF-DNBS with HSA. Stock solutions of **KF** and **KF-DNBS** were prepared in DMSO, and a stock solution of HSA was prepared in distilled water. Blanks, each containing only **KF** or **KF-DNBS** (25 μM, 10% DMSO), and samples, containing each **KF** or **KF-DNBS** (25 μM, 10% DMSO) with HSA (100 μM) in sodium phosphate buffer (SPB, pH 7.4, 20 mM), were prepared. Fluorescence spectra were then recorded using the fluorescence spectrophotometer under excitation at 420 nm.

Optimization of Sensing Conditions for Selective H_2S Detection over Cys and Hcy. The samples containing HSA (100 μM) and each biothiol (H_2S 100 μM, Cys 250 μM, Hcy 100 μM, GSH 10 μM) with and without 2-FBBA (2 mM) in SPB (pH 7.4, 20 mM) were incubated for 15 min at 25 °C. **KF-DNBS** (25 μM, 10% DMSO) was added to the samples followed by the measurement of fluorescence spectra under excitation at 420 nm at 37 °C.

Determination of the Limit of Detection (LOD) for H_2S. Fluorescence spectra of **KF-DNBS** (25 μM, 10% DMSO) with HSA (100 μM) containing various concentrations of H_2S (0, 5, 10, 20, 40, 60, 80, 100, 150, 250 μM) in SPB (pH 7.4, 20 mM) were recorded under excitation at 420 nm for 40 min at 5 min intervals at 37 °C. The experiment was repeated three times. The LOD was calculated using 3σ/slope based on the titration experiment, in which σ is the standard deviation of the blank measurements and the slope value is obtained from a plot of the fluorescence intensity versus H_2S concentration.

pH Dependency of KF-DNBS with HSA upon H_2S. Samples containing HSA (100 μM) and 2-FBBA (2 mM) without and with H_2S (100 μM) in different buffer conditions (pH 5 acetate, pH 6−8 SPB, pH 9 Tris; 20 mM) were incubated for 15 min at 25 °C. **KF-DNBS** (25 μM, 10% DMSO) was added to the samples followed by the measurement of the fluorescence intensity at 500 nm after 10 min under excitation at 420 nm at 37 °C. The experiment was repeated three times.

Selectivity of KF-DNBS toward H_2S over Other Biological Analytes. A blank containing no analyte and a sample containing each analyte (H_2S 100 μM, Cys 250 μM, Hcy 100 μM, GSH 10 μM, HSO_4^- 100 μM, SO_4^{2-} 100 μM, SO_3^{2-} 100 μM, $S_2O_3^{2-}$ 100 μM, SCN^- 100 μM, CN^- 100 μM, F^- 100 μM, Br^- 100 μM, NO_3^- 100 μM, NO_2^- 100 μM, HCO_3^- 100 μM, $CH_3CO_2^-$ 100 μM, H_2O_2 100 μM, ClO^- 100 μM) were prepared followed by the addition of HSA (100 μM) and 2-FBBA (2 mM) in SPB (pH 7.4, 20 mM). After incubation for 15 min at 25 °C, **KF-DNBS** (25 μM, 10% DMSO) was added to each sample, and then fluorescence intensity at 500 nm was recorded using the fluorescence spectrophotometer under excitation at 420 nm at 37 °C. The experiment was repeated three times.

Quantitative Detection of Spiked H_2S in Human Serum. Human serum samples spiked with different concentrations of H_2S (25, 50, 100, 150 μM) were prepared. A sample containing HSA (100 μM), 2-FBBA (2 mM), and H_2S-spiked serum sample (20%) in SPB (pH 7.4, 20 mM) was incubated for 15 min at 25 °C. **KF-DNBS** (25 μM, 10% DMSO) was added to the sample, and the fluorescence intensity at 500 nm was recorded using the fluorescence

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spectrophotometer under excitation at 420 nm at 37 °C. The recovery was calculated based on the standard curve obtained from H_2S titration. The experiment was repeated three times.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04659.

> Fluorescence-based screening of **KF** against major serum proteins; comparison of the fluorescence change of **KF** and **KF-DNBS** with HSA; photo/thermostability of **KF** and **KF-DNBS**; quantum yields of **KF** and **KF**-HSA complex; kinetics of the reaction of 2-FBBA with biothiols (H_2S, Cys, Hcy, GSH); absorption spectral change of the probe **KF-DNBS** to H_2S; mechanism studies; competition study; calibration curve for quantitative analysis of H_2S in real samples; ^1H, $^{13}C\{^1H\}$ NMR, and HRMS spectral data (PDF)

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Notes

The authors declare no competing financial interest.

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