Fluorescent Probes

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A Fluorescent Probe for Fast and Quantitative Detection of Hydrogen Sulfide in Blood**

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Hydrogen sulfide (H_2S), well-known for its unpleasant rotten egg smell, was traditionally considered as a toxic gas. However, recent studies have demonstrated that hydrogen sulfide is an endogenously produced gaseous signaling compound (gasotransmitter) with importance on a par with that of the other two known endogenous gasotransmitters, nitric oxide (NO)[1] and carbon monoxide (CO).[2] H_2S has been recognized for mediating a wide range of physiological effects. Studies have shown that H_2S can have an effect on the cardiovascular system[3] by acting as a K-ATP channel opener.[4] Several studies have shown the protective roles of H_2S, in situations such as myocardial ischemia, most likely through a combination of its antioxidant and anti-apoptotic signaling effects.[5] Further studies also showed that H_2S may be of therapeutic benefit for the treatment of ischemia-induced heart failure.[6,7] It is also a modulator in the central nervous system,[8–10] respiratory system, gastrointestinal system, and endocrine system.[11] It seems that hydrogen sulfide exhibits almost all the beneficial effects of NO without generating the toxic reactive oxygen species (ROS). In addition, it also acts as an antioxidant or scavenger of ROS. Furthermore, research has indicated that the hydrogen sulfide level is related to diseases such as the Down syndrome[12] and Alzheimer's disease.[13] Therefore, recent years have seen a steady increase in interest in understanding the physiological and pathological functions of hydrogen sulfide.[11,14,15] One significant limiting factor in studying hydrogen sulfide is the lack of sensors and agents that allow for its rapid and accurate detection. Methods using colorimetric,[16–18] electrochemical analysis,[19–21] and gas chromatography have been reported.[22,23] However, catabolism of hydrogen sulfide is known to be fast, which could result in continuous fluctuation in its concentration, leading to difficulties in the accurate analysis of this important molecule. Current methods do not allow for fast, accurate, and real-time determination. Different endogenous sulfide concentrations have been reported with most publications suggesting the sulfide concentration in blood is in the range of 10–100 μM.[24–29] There have been other studies suggesting much lower sulfide concentrations.[30,31] Therefore, new methods are needed for the efficient detection of sulfide in biological systems.

With the idea of developing a new method that will be useful for rapid assay of hydrogen sulfide concentrations under physiological conditions, we undertook the effort of searching for a selective chemosensing agent for this important gasotransmitter. For easy use in a biology laboratory, the chemosensing agent should 1) act fast (within seconds) under mild conditions, 2) be chemically stable for long-term storage, 3) be sensitive for detection under near physiological conditions, 4) show a linear concentration–signal relationship within physiologically relevant hydrogen sulfide concentration ranges for easy quantitation, 5) show minimal or no interference by other anions in the blood, and 6) be functional in aqueous solutions and blood plasma. Herein, we report the development of a fluorescent chemoprobe and its application in the determination of hydrogen sulfide in aqueous solution, serum, and whole blood.

Fluorescence is one of the most sensitive detection methods. Thus we were interested in selecting a fluorophore, which has a high quantum yield, emits at a long wavelength, and responds to hydrosulfide by fluorescent property changes. Dansyl is a commonly used fluorophore, and well-known for its strong fluorescence and long emission wavelength. We were interested in designing a sulfide-sensitive agent using this fluorophore by taking advantage of the known unique reduction of an azido group by hydrogen sulfide.[32] We reasoned that the reduction of an azido group attached to a strongly electron-withdrawing group would occur at an accelerated rate. Because of the difference in electronegativity of the azido and amino groups and the added degree of rotational freedom for the azido group, reduction of sulfonyl azide into sulfonamide should trigger a change in the electronic properties and thus the fluorescent properties of the dansyl moiety.[33] Therefore, we synthesized dansyl azide (DNS-Az, **2**, Scheme 1).[34]

DNS-Az (**2**) by itself is nonfluorescent. However, upon addition of hydrogen sulfide the DNS-Az solution showed a strong fluorescence enhancement, as expected (Na_2S was

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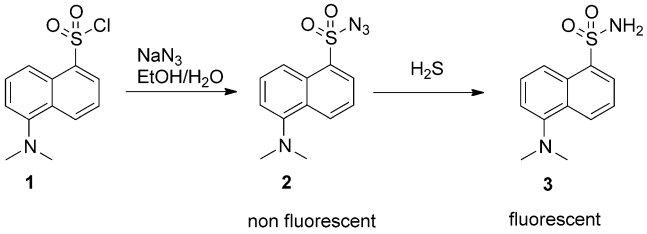

Scheme 1. DNS-Az (**2**) as a fluorescent probe for sulfide.

used as a hydrogen sulfide source in all experiments). The addition of 25 μM hydrogen sulfide led to a 40-fold fluorescence enhancement in 20 mM sodium phosphate buffer (pH 7.5) with 0.5 % Tween-20 (Buffer/Tween). The detection limit was as low as 1 μM with a signal-to-noise ratio (S/N) of 3:1. HPLC, MS, and NMR analysis confirmed that the fluorescence increase was due to the formation of dansyl amide (**3**, Scheme 1, see the Supporting Information for spectral data).

To study the selectivity of this chemoprobe for sulfide, the fluorescent properties of DNS-Az (**2**) in the presence of various anions were examined in buffer/Tween. No comparable response was observed from other anions (Figure 1). Because the detection is based on the reducing properties of sulfide, other possible reducing anions such as iodide, bromide, fluoride, bisulfite, and thiosulfate were also tested. Overall 18 anions were screened and no obvious response was observed for most of the anions at a concentration of 1 mM, which is a 40-fold higher concentration than that of sulfide. The same results were observed for Cl^- at 150 mM, HCO_3^- and citrate at 100 mM, which are equal to or higher than the physiological concentrations of these anions. None of the other anions should have a concentration higher than 1 mM under normal physiological conditions. Among all the anions, only HSO_3^-, $S_2O_4^{2-}$, and $S_2O_5^{2-}$ led to some fluorescent intensity increases. However, the extent of the fluorescence increase was far smaller than that caused by sulfide even when the concentrations of those anions were four-fold higher than that of sulfide (Figure 2b, for details see Figure S5 in the Supporting Information). The response of DNS-Az (**2**) to other reducing agents, such as thiophenol, benzyl mercaptan,

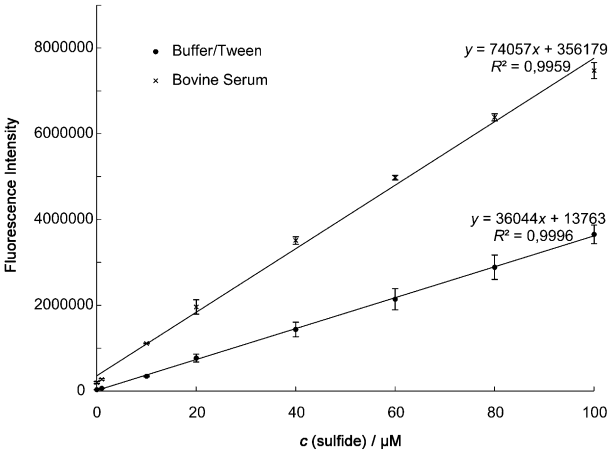

Figure 2. Hydrogen sulfide concentration-dependent fluorescence intensity changes determined using 96 well plates: DNS-Az 200 μM, Na_2S 0–100 μM in commercial bovine serum and buffer/Tween (excitation filter 340 nm, emission filter 535 nm).

and cysteine was also tested. Benzyl mercaptan was the only compound that induced strong responses (about 1/5 of that of sulfide, see Figure S11 in the Supporting Information), which could pose an interference problem. However, this finding should not be a practical issue because benzyl mercaptan is rarely found in biological systems. We also found that DNS-Az was recalcitrant to the possible displacement reaction resulting from attack by an amino group. DNS-Az showed very limited response to glycine and lysine at concentrations as high as 50 mM (see Figure S12 in the Supporting Information).

A linear relationship is always important for easy and accurate analysis. Thus, the dependence of fluorescent changes on hydrogen sulfide concentration was studied using both a fluorometer and a microplate reader. DNS-Az (**2**) reacts with sulfide essentially quantitatively even in aqueous solution. The fluorescence intensity showed a reproducible linear relationship in buffer/Tween against hydrogen sulfide (Figure 2). When the sulfide concentration is higher than that of DNS-Az (**2**), the plot was found to reach a plateau (see Figure S9 in the Supporting Information), which means that the stoichiometry of this reaction was 1:1.

Thus far, all the selectivity and linearity studies suggest that DNS-Az (**2**) can be used for the determination of sulfide

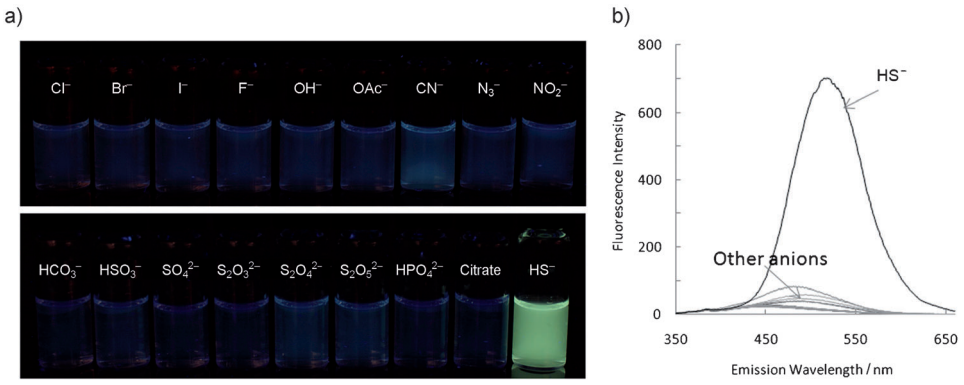

Figure 1. a) Fluorescence intensity changes of DNS-Az (**2**) in solution after addition of sulfide and other anions. b) Fluorescence spectra of DNS-Az (**2**) upon addition of various anions (DNS-Az 100 μM, Cl^- 150 mM, HCO_3^-, citrate 100 mM, HSO_3^-, $S_2O_4^{2-}$, and $S_2O_5^{2-}$ 100 μM as well as HS^- 25 μM and all other anions at 1 mM in 20 mM sodium phosphate buffer (pH 7.5) with 0.5 % Tween-20, $\lambda_{ex} = 340$ nm). Anions tested: Cl^-, Br^-, I^-, F^-, OH^-, OAc^-, CN^-, N_3^-, NO_2^-, HCO_3^-, HSO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, $S_2O_4^{2-}$, $S_2O_5^{2-}$, HPO_4^{2-}, and citrate.

concentrations in a biological sample. For a further test, DNS-Az (**2**) was evaluated in commercially available bovine serum. Upon addition of sulfide, the solution of **2** in serum showed very significant fluorescent intensity increases. Though bovine serum showed background fluorescence (Figure 3

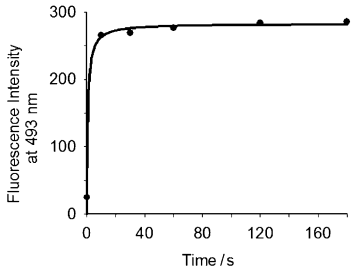

Figure 3. Reaction time profile of DNS-Az (**2**) and hydrogen sulfide (DNS-Az 100 μM, H_2S 30 μM in bovine serum).

and Figure S8 in the Supporting Information at 0 s), it was negligible compared to the strong fluorescence of dansyl amide (**3**) obtained from the reaction (Figure 3). It should be noted that the reaction went very fast in bovine serum (complete within seconds, Figure 3 and Figure S8 in the Supporting Information). This is very important considering the fast metabolism and volatile nature of hydrogen sulfide in biological systems. This unprecedented fast response could provide the possibility of quantitative detection without any pretreatment of samples. The reaction time profile was studied in different solvent systems (see Figures S6 and S7 in the Supporting Information).

An excellent linear relationship was also obtained in bovine serum (Figure 2). The standard curve covers the range of reported endogenous levels of hydrogen sulfide, indicating that this probe is very suitable for the detection of sulfide in biological samples. Due to changes in the microenvironment and viscosity of the medium, fluorescence intensity and emission wavelength vary when different media are used. A standard addition procedure (internal spiking) was used for accurate measurement of samples when the medium was not readily available for generation of a calibration curve. Overall, the results suggest that the anions and biological substrates normally encountered in the blood do not pose a problem in the quantitative detection of hydrogen sulfide in a biological sample.

Encouraged by the promising results obtained in buffer and bovine serum, we applied this fluorescent chemoprobe to the determination of hydrogen sulfide concentrations in blood using the C57BL6/J mouse model (Figure 4 and Figure S3 in the Supporting Information), which we have been using in other related studies. A standard addition procedure was used in the experiment. Five mice were used in this study (Table 1). The average sulfide concentration in these five mice was (31.9 ± 9.4) μM, very similar to previous reported values in mouse plasma (34.1 μM).[27] This has confirmed that our fluorescent chemoprobe can indeed be used in the detection of hydrogen sulfide in real biological samples.

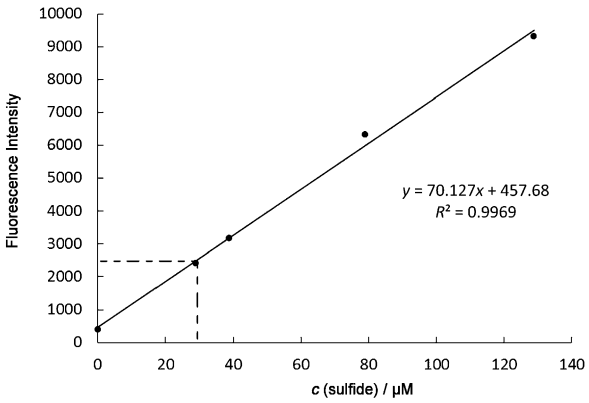

Figure 4. Determination of the hydrogen sulfide concentration in blood using C57BL6/J mice (see Table 1) and 96 well plates (DNS-Az 200 μM, excitation filter 360 nm, emission filter 528 nm). The zero point was obtained by trapping sulfide with $ZnCl_2$ and the calibration curve was obtained by using an internal standard method. (Hydrogen sulfide concentrations 0, *x*, *x* + 10, *x* + 50, and *x* + 100 μM).

Table 1: Measurement of hydrogen sulfide concentrations in mouse blood.

Mouse	$c(H_2S)$ [μM]
1	25.0
2	30.2
3	26.1
4	48.4
5	28.9
Average	**31.9 ± 9.4**

In conclusion, a novel reduction-sensitive fluorescence chemoprobe was developed for hydrogen sulfide detection in aqueous solutions, including blood serum and whole blood. The probe was found to be very selective for sulfide among 18 anions tested and other common reducing species, with a detection limit of 1 μM in buffer/Tween and 5 μM in bovine serum with a signal-to-noise ratio of 3:1. The linear relationship obtained in bovine serum covers the reported endogenous concentration range of hydrogen sulfide. The probe was used in the detection of hydrogen sulfide in blood using the C57BL6/J mouse model. The result (31.9 ± 9.4 μM) was very close to the previously reported serum concentration of hydrogen sulfide. In addition, this agent is characterized by simplicity and ease in measurements, and compatibility with 96 well plates and microplate reader, which are readily accessible in biology labs. Hydrogen sulfide concentrations in biological systems are tightly regulated and can experience rapid changes. The unprecedented fast response by DNS-Az (**2**) to sulfide allows it to be used for the detection of transient changes in hydrogen sulfide levels without sample pretreatment. The probe, DNS-Az (**2**), is simple in structure, very easy to synthesize, stable, and amenable to long-term storage. A new research field has emerged in the past 15 years because of the newly recognized significance of hydrogen sulfide as an endogenous gasotransmitter. The molecular mechanism of cellular actions of sulfide remains to be understood, and novel H_2S-releasing drugs need to be developed. We feel that this

fast, selective, efficient, and low-cost detection method for sulfide will be very useful in the booming research field of hydrogen sulfide.

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