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Reversible Detection and Quantification of Hydrogen Sulfide by Fluorescence using the Hemoglobin I from *Lucina Pectinata*.

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ABSTRACT: A new detection system for the endogenous gaseous transmitter and environmental pollutant hydrogen sulfide is presented. It is based on the modulation of the fluorescence spectrum of a coumarin dye by the absorption spectrum of the recombinant hemoglobin I from clam *Lucina Pectinata* upon coordination of the analyte. While we establish that the reported affinity of rHbI for H_2S has been overestimated, the association of the protein with an appropriate fluorophore allows fast, easy and reversible detection and quantification of hydrogen sulfide in buffer as well as biological fluids such as human plasma, with a quantification limit around 200 nM at pH 7.4.

The demonstration by Snyder *et al.*[1] that hydrogen sulfide (H_2S) is endogenously produced and acts as a vasodilator in mammals was a turning point in the history of this gas in biology. Indeed, this work confirmed the hypothesis that emerged in the late 90's that H_2S could be the third gaseous transmitter, along with nitric oxide NO and carbon monoxide CO.[2] It also boosted the development of techniques and molecular tools for studying the potential therapeutic role of H_2S, and its signaling functions. Thus, numerous hydrogen sulfide donors mimicking the slow continuous biosynthesis of hydrogen sulfide were designed.[3, 4] Simultaneously, various methods for measuring H_2S in biological media were developed. At first, these methods were mostly derived from procedures used in environmental chemistry, since sulfur is a major physicochemical and geological indicator and hydrogen sulfide a natural and anthropogenic pollutant, but they were not appropriate for biological studies. For instance, these methods resulted in aberrant concentrations of hydrogen sulfide in biological fluids.[5] Later, more relevant techniques, using fluorescence, polarography or chromatography, were developed to detect or quantify H_2S in biological samples. However, each offers its own advantages and disadvantages.[6, 7] For instance, chromatography displays a detection limit ~ 1 nM, but requires a derivatization step and a chromatographic separation, making it time consuming but well suited for analytical studies requiring high sensitivity. Polarographic sensors allow for real-time detection of hydrogen sulfide,[8] but they require costly apparatus. Last, fluorescent sensors are nowadays the best choice for bioimaging H_2S.[9] However, they are not suitable for real time detection of H_2S, because of their reaction kinetics and lack of reversibility. Indeed, most specific fluorescent sensors are slow "reaction based" probes, which consume the analyte. Typical strategies include the reduction of azides[10] or nitro compounds[11], the use of the dual-nucleophilicity of H_2S,[12] or the formation of metal sulfides.[13] On another hand, several approaches using non-covalent interactions are currently explored to develop reversible detection systems. For instance, the reversible adsorption/desorption of H2S on molybdenum oxide was used to detect gaseous hydrogen sulfide.[14] Pluth *et al.* recently reported a UV-absorbing supramolecular receptor that binds the hydrosulfide anion in organic solvents. Additionally, we and others are aiming at the development of reversible coordination-based fluorescent sensors for H2S in aqueous buffered solutions. However, these systems so far suffer from moderate affinities and / or detection limits. [15, 16, 17] Here, we present a new selective and reversible fluorescent system based on the "absorption into fluorescence", a strategy recently introduced by D'Auria *et. al.*,[18] to selectively detect hydrogen sulfide in real time with competitive detection limits of ~ 200 nM at pH 7.4. This strategy consists in using a sensor with a high molar attenuation coefficient to modulate the fluorescence properties of a fluorophore. To be efficient, the absorption band of the sensor should overlap with the excitation band of the fluorophore, and it should shift upon reaction with the analyte. As such, we selected the most avid biological system for hydrogen sulfide as a sensor, *i.e.* the hemoglobin HbI for the clam *Lucina Pectinata*.[19] The unique affinity of HbI for H_2S lies in the peculiar layout of its distal active site which contains a glutamine and two phenylalanines at the E7, B10 and E11 positions, respectively.[20] These unusual structural determinants allow for fast binding and slow dissociation of the protonated form H_2S to the ferric heme center of HbI.[19, 21] Despite the high affinity displayed by HbI for hydrogen sulfide ($K'^{\dagger}_{d}$ = 3.4 nM),[19] its use has so far been limited to the determination of H_2S solubility by spectrophotometry,[22] or to the tentative attempt to prepare a bioelectrode.[23] In addition to its high affinity for H_2S, HbI is also perfectly suited for the biomolecular gating approach reported therein that necessitates a strong chromophore. Indeed, upon the coordination of H_2S, the observed red-shift of the Soret band (ε ~ 178 000 $L.mol^{-1}.cm^{-1}$) from 407 to 425 nm should results in a measurable modulation of the photophysical properties of a fluorophore with an appropriate excitation around 407 nm.

EXPERIMENTAL SECTION

Materials. Restriction enzymes and Quick-Ligation kits were purchased from New England Biolabs. Terrific broth medium (Sigma EZmixT9179) and hemin (Sigma 51280) were purchased from Sigma-Aldrich. Kanamycin and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from Euromedex. The BLi5 strain was a gift from Jacques Ballalou (Institut Pasteur, Paris, France). The Protein 200 Plus reagent kits and chips were provided by Biorad. Ultrafiltration devices Amicon Ultra-15 were obtained from Millipore. Anhydrous sodium hydrosulfide (NaSH) was purchased from Alfa Aesar, and kept in a glove box until use.

Solutions were prepared in buffer A previously degassed by purging with argon.

The synthesis of N-[2(2-Hydroxyethoxy)ethyl]-6,8-difluoro-7-hydroxycoumarin-3-carboxamide was carried out in DMF from pacific blue succidimidyl ester (ThermoFisher scientific), 2-(2-aminoethoxy)ethanol and triethylamine (Aldrich) as previously described. [24]

All reactions were carried out in buffer A: potassium phosphate buffer (KPi) (50 mM, pH 7.4 supplemented with 1 mM of diethylenetriaminepentaacetic acid (dtpa) as a chelating agent), unless stated otherwise.

Cloning of HbI ORF into the pET28a expression vector. The cDNA sequence of HbI from *Lucina Pectinata* [25] was obtained from Genbank database (GenBank: AF187049.1) and the rare codons changed to optimize expression in host *E. coli*. A NcoI restriction site was added in frame at the 5' end to facilitate cloning. The modified sequence was synthesized and cloned into a pUC57 vector by Proteogenix (Schiltigheim, France). The pUC57-HbI vector was then digested by NcoI / BamHI, and the resulting gel-purified inserted sequence NcoI/BamHI HbI cDNA quick-ligated into the pET28a expression vector previously digested by NcoI / BamHI, and dephosphorylated. The resulting pET28a-HbI vector was used to over-express the recombinant rHbI.

Expression of recombinant rHbI in *E. coli* BLi5 strain. The culture protocol was derived from Leon *et al.*[26] with modifications as follows. The vector pET28a-HbI was transformed into BLi5 *E. coli* strain for protein expression. An overnight culture was added at 1 / 50 volume to Terrific broth medium supplemented with Kanamycin 100 µg/mL and chloramphenicol 30 µg/mL. Bacteria were grown at 37°C until OD_{600} = 2. After lowering the temperature to 30°C, hemin (30 µg/mL) and IPTG 1 mM were added to induce rHbI expression. After 16 h culture at 30°C and 150 rpm, bacteria were pelleted and stored at −80 °C until use.

Purification of recombinant rHbI. Bacterial pellets were thawed and re-suspended in 50 mM Hepes buffer (pH 7.5) containing Complete™ protease inhibitor cocktail (Roche) and DNaseI (60 µg /mL). After sonication of the solution with a Vibra-cell sonicator (Fisher Bioblock, Vibra-Cell 75115, 500 W) at 30 % amplitude for 6 min on ice (3 s on, 15 s off), the solution was centrifuged (18 000 rpm; 37 600 g for 45 min), and the clarified supernatant was then subjected to ammonium sulfate precipitation (0→55→90 %) and then centrifuged (15 000 rpm; 14 000 g) for 20 min at 4 °C. The 55→90% pellet was re-suspended and dialyzed overnight against 20 mM Tris.HCl buffer (pH 8.5) at 4°C, followed by two buffer changes. The dialysate was incubated in batch with Q-sepharose resin (1 mL for 15 mg proteins) for 1 h 30 at 4°C. The Q-sepharose resin was then washed with 3 volumes of 20 mM Tris-HCl buffer (pH 8.5) at 4°C and the flow-through containing rHbI was concentrated with an Amicon ultra-15 mL filter (3 kDa cut-off). The solution was then loaded on a HiLoad 16/60 Superdex-75 column (GE Healthcare) equilibrated with 25 mM Hepes buffer (pH 7.5), 150 mM KCl, at a flow rate of 0.3mL/min. After analysis by microfuidic electrophoresis with Experion (Biorad), fractions containing pure rHbI were pooled, concentrated with an Amicon ultra-15 mL filter, and stored as aliquots at -80°C. Typical yields were 30-40 mg HbI per L of culture. According to Experion analysis (Biorad), the purity of rHbI was ≥95 %. The molecular mass of 15835 Da, assessed with the calibrated Superdex-75 was close to the theoretical value for the monomeric holoprotein (15429 Da). The molecular mass determined by ESI-MS on an LTQ Orbitrap instrument (14812.4 Da) confirmed to be in good agreement with the theoretical value for the apoprotein (14812.8 Da).

Physical measurements. UV-vis spectra were recorded on a Shimadzu UV-2700 or Carry 300 at 25°C using 1 mL or 100 µL quartz cuvettes. Stopped-flow kinetic experiments were performed on a Biologic SFM-300 at 303 K. Fluorescence spectra were recorded on a Hitachi F-7000 spectrometer at 25°C using 100 µL quartz cuvettes. Excitation wavelength at 410 nm and emission wavelength at 455 nm were used. All kinetics and thermodynamic parameters were obtained by fitting the experimental data with the appropriate equation using SigmaPlot.

Kinetics studies. A) For stopped flow studies, two syringes (S1 & S2) were used, with the following contents: S2: NaSH at various concentrations in buffer A, S1: 6 µM solution of rHbI in buffer A. Each run consisted of mixing 1/1 parts of S1/S2. An average of at least three runs was recorded for each substrate concentration. B) The determination of the association rate constant k'_{on} for the fixation of the cyanide (0.025-0.375 mM) to rHbI was determined at 25°C by UV/Vis spectroscopy by monitoring the change in absorbance at 420 nm as a function of time. The data were fitted with an exponential equation to obtain the k'_{obs} value at each concentration of cyanide. The k'_{obs} values were then plotted against the concentrations of potassium cyanide to get the k'_{on} and k_{off} value by fitting the data with a linear equation. C) The determination of the k_{off} of rHbI- H_2S was carried out by UV/Vis spectroscopy. Typically: 5 µL of a 1.2 mM solution of rHbI were mixed in 935 µL of buffer A with 60 µL of a 1 mM solution of NaSH. A first UV/Vis spectrum was recorded to confirm the formation of rHbI- H_2S, then 10 µL of a 2 M solution of potassium cyanide (20 mM final) were added to the reaction mixture and the displacement reaction was monitored by UV/Vis is spectroscopy.

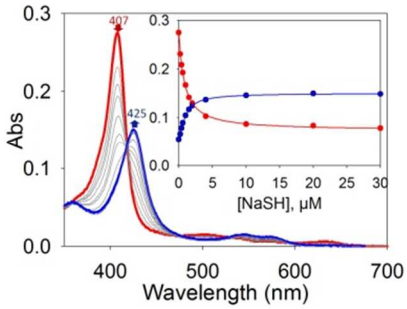
Fluorescence studies. A typical experiment is as follows: to a 100 µL cuvette were added 97 µL of buffer A, 1 µL of a 100 µM solution of PB in DMSO (1 µM final), 2 µL of a 1 mM solution of rHbI in buffer A (20 µM final). The average value of three recordings was used as the initial fluorescence value (F_0). For each concentration, the average of three measurements was used to determine the ratio F/F_0. Each experiment was independently repeated 3 times to determine the S.D..

Plasma studies. Plasma from whole blood samples from healthy blood bank donors ("Etablissement Français du Sang", Convention # 07/CABANEL/106; Paris; France) was obtained by density centrifugation.

RESULTS

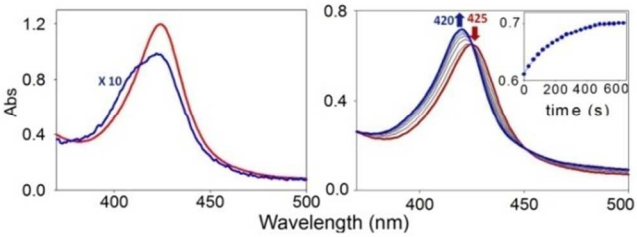
Characterization of rHbI and reactivity with H_2S. The recombinant protein rHbI was produced in the BLi5 *E. coli* strain, after optimization of the protocol first described by López-Garriga *et al.*[26] Derived from the BL21(DE3), this strain harbors a plasmid pDIA17[27] coding for the lacIq repressor, which maintains the recombinant protein expression strongly repressed until it is induced by IPTG. In addition, the BLi5 strain also efficiently incorporates hemin from the culture medium.[26] Briefly, after optimization of the cDNA sequence[25] (mainly the codon usage), the later was synthesized and cloned into a pU57 vector. Following a subcloning step into a pET28a expression vector, the BLi5 *E. coli* strain was transformed with the resulting pET28a-rHbI vector and rHBI protein expression was induced by addition of IPTG in the presence of hemin. The oxy-ferrous form of the protein, which displays the expected mass for the monomeric rHbI (see Experimental part), was then obtained after salting out and two chromatographic steps with typical yields of 30-40 mg rHbI per L of culture and a purity > 95%.

Figure 1. UV-visible titration of a 1.5 µM solution of rHbI in buffer A (50 mM KPi, pH 7.4, 1 mM dtpa) by sodium hydrosulfide.



The ferric form of rHbI was obtained upon oxidation with ferricyanide of the purified oxy-ferrous form of rHbI. It displays a UV/Vis spectrum identical to the one reported for the wild type HbI isolated from the clam or the recombinant HbI.[19, 26] Upon addition of an excess of sodium hydrosulfide, the initial Soret and Q bands of the met-aquo form of rHbI (407, 502 and 629 nm) shift to new values (359, 425, 545 and 575 nm) corresponding to the met-hydrogensulfide form rHbI-H_2S (Figure S1). The formation of the later was also corroborated by resonance Raman spectroscopy (data not shown), which indicates a spin change from 5/2 to 1/2 (the marker ν_3 shifts from 1483 cm^{-1} to 1500 cm^{-1}) and the formation of a Fe-S bond ($\nu_{Fe\text{-}S}$ = 374 cm^{-1}).[28] To confirm that the binding properties of rHbI are identical to the ones displayed by wtHbI, we determined the relative kinetic constants k'_{on}[†] and k_{off} for its interaction with H2S. Stopped-flow absorbance experiments performed under pseudo-first order conditions allowed the determination of a k'_{on} = (57.6 ± 2.0) x 10^3 $M^{-1}.s^{-1}$ (Figure S2). Monitoring of the exponential decay (or rise) of the absorbance at 425 (or 407) nm after dilution of a concentrated solution of rHbI-H_2S (Figure S3) gave k_{off} values of (2.5 ± 0.1) x 10^{-4} s^{-1} and (2.1 ± 0.1) x 10^{-4} s^{-1}, respectively. These data correlate very well with those reported for the wild type enzyme (k'_{on} ~ 65 x 10^3 $M^{-1}.s^{-1}$ and k_{off} = 2.2 x 10^{-4} s^{-1}, respectively).[19] Calculation of the dissociation constant from these values gives K'$_d$ values of 3.8 nM and 3.4 nM for rHbI and wtHbI, respectively. It has been shown that the determination of the dissociation constant under thermodynamic conditions by a titration experiment resulted in a K'$_d$ = 90 nM for wtHbI.[19] We have performed a similar experiment with rHbI, during which we followed the increase in absorbance at 425 nm or the decrease at 407 nm observed upon addition of sodium hydrosulfide to rHbI (Figure 1). The corresponding data were fitted with the quadratic equation (1) (Figure S4), giving rise to K'$_d$ values of 332 ± 32 and 347 ± 59 nM at 425 and 407 nm, respectively. These values are within the same range as the 90 nM reported for wtHbI, although larger, most probably because of different experimental settings and of the use of sodium hydrosulfide instead of the hydrogen sulfide gas. Noteworthy, these values strongly differ from the low nM affinities obtained from the kinetic data.

Figure 2. Left: UV/Vis spectra of an 11 µM solution of rHbI in Buffer A with 110 µM NaSH, recorded before (red) and immediately after (blue) dilution. For comparison purpose, the UV/Vis spectrum of the later curve has been multiplied by 10. Right: monitoring of the evolution of the UV/Vis spectra of a 6 µM solution of rHbI-H_2S (from 6 µM rHbI and 60 µM NaSH) after addition of a 20 mM solution of potassium cyanide. Inset: monitoring of the absorbance at λ = 420 nm over time.



At first, we assumed that the origin of the aforementioned discrepancies originated from an overestimated assessment of the k_{off}. Accordingly, we decided to focus on the dissociation step, and to begin with, we recorded the UV/Vis spectra of a solution of rHbI-H_2S before and rapidly after its dilution. The results shown in Figure 2 (left) indicate that approximately 15% of rHbI-H_2S has been converted to rHbI within the mixing time, which is clearly inconsistent with the half-life of 3150 s determined from the dissociation rate constant k_{off}. Based on this experiment, we posited that a displacement experiment in the presence of cyanide as the competing ligand would be appropriate to obtain a more definite dissociation rate constant value.[29] Therefore, we next monitored the conversion of rHbI-H_2S to rHbI-CN over time by UV/Vis spectroscopy at 420 nm (Figure 2 right). It follows pseudo-first order kinetics, and the dissociation rate constant for hydrogen sulfide was calculated with equation (2) (Figure S5), yielding a k_{off} = (7.5 ± 2.5) x 10^{-3} s^{-1}. This new value explains nicely the experiment described in Figure 2 (left). It also rationalized the previously high K'$_d$ values determined by titration experiments with rHbI and wtHbI (332 and 90 nM, respectively), which are now in the range of those calculated by the ratio k_{off} / k'_{on} (131 nM and 115 nM, respectively).

Using HbI to detect H_2S by fluorescence. With the idea to use the "absorption into fluorescence" strategy for developing a turn-on fluorescent sensor to detect hydrogen sufide, we selected N-[2(2-Hydroxyethoxy)ethyl]-6,8-difluoro-7-hydroxycoumarin-3-carboxamide, a derivative[24] of the coumarin dye "Pacific Blue™" (PB[‡]), as fluorophore. We anticipated that the intense absorption of the Soret band of rHbI at 407 nm would efficiently filter the light at the maximum excitation wavelength of the coumarin (404 nm), and turn its fluorescence off in the absence of the analyte hydrogen sulfide. Because of the red-shift (18 nm) and the decrease in the attenuation coefficient associated with H_2S binding to rHbI (178000 *vs* 102000 $L.mol^{-1}.cm^{-1}$, respectively),[22] the fluorescence should be partially restored in the presence of the sulfur-containing analyte, as schematized in Figure 3. To confirm our aforementioned hypothesis, we first added increasing amounts of rHbI to a buffered solution of the fluorophore (Figure S6). As expected, this leads to a decrease in the fluorophore's fluorescence intensity, both in the emission and excitation spectra (Figure S6). For instance, a 35-fold decrease in the excitation and emission spectra of a 1 µM solution of the fluorophore is observed in the presence of 20 µM of rHbI.

Figure 3. Principle of the turn-on fluorescent system proposed in our work: the sensor rHbI plays the role of a filter at the excitation wavelength of fluorophore in absence of H_2S, but not in its presence.

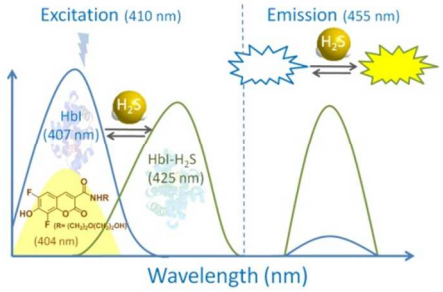
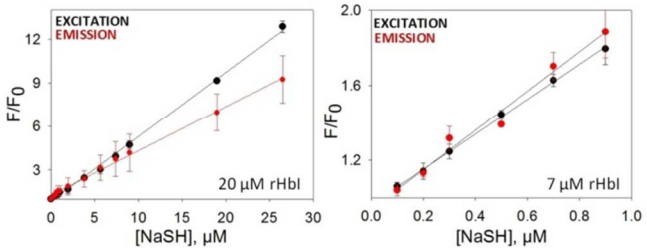


Figure 4. Fluorescence ratio and corresponding standard deviations observed upon addition of NaSH to a 1 µM solution of fluorophore containing either 20 µM (right) or 7 µM rHbI (left) in buffer A (50 mM KPi, pH 7.4, 1 mM dtpa) containing 0.9 % DMSO). Excitation wavelength: 410 nm / Emission wavelength: 455 nm. Data are represented as the mean ± S.D. (n=3).



Next, we added sodium hydrosulfide to the previous solution to decipher if the presence of the analyte partially restores the fluorescence of the fluorophore. As expected, the addition of NaSH immediately leads to a substantial increase in the fluorescence intensity (Figure 4). To substantiate the principle pictured in Figure 3, we first ruled out the direct reaction between PC and sodium hydrosulfide as a potential explanation for the gain of fluorescence intensity (Figure S7). We also confirmed that the red-shift of the Soret band of rHbI upon coordination of hydrogen sulfide occurs in the presence of PC (Figure S8). Finally, we observed that the substitution of rHbI by met-myoglobin (which possesses a Soret band at 408 nm but does not tightly binds hydrogen sulfide) results in a quenching of the fluorescence of PC, but not to its restoration in the presence of sodium hydrosulfide (Figure S9). All these data indicates that our system indeed operates by "absorption into fluorescence".

To better define it, we performed experiments with a fixed concentration of the fluorophore PB (1 µM) and various rHbI and NaSH concentrations. For sodium hydrosulfide concentrations higher than 1 µM, we found that 1 µM of PB and 20 µM of rHbI allows a linear quantification of up to 25 µM sodium hydrosulfide (Figure 4 left). At saturation, the maximum recovery of fluorescence was a 17-fold increase in intensity. For sub-micromolar concentrations of sodium hydrosulfide (Figure 4 right), we observed that a lower concentration of the heme sensor (7 µM) was better suited. Under these conditions, we determined quantification limits (calculated as being 10 time the standard error / slope) of 226 and 294 nM for sodium hydrosulfide, using excitation and emission spectra, respectively. It must be noted that rHbI is specific for the protonated form of the analyte H_2S, and that the aforementioned values are likely to be reduced at lower pH, as the affinity of rHbI for the analyte will increase ($K_d = [K'_d/(1+(10^{-pKa}/10^{-pH})]$, with pKa = 6.8 for H_2S).

Next we investigated the selectivity of our detection system. As shown in Figure 5 (left), our system is selective for hydrogen sulfide, since no significant variation of the fluorescence intensity is observed in the presence of physiological concentrations of glutathione (5 mM), cysteine (100 µM), or other possible interfering species. Prominently, our sensor system is reversible, as depicted in Figure 5 (right). Indeed, after addition of a saturating concentration of sodium hydrosulfide to our gating system, the initial fluorescence ratio is recovered within minutes by carefully bubbling argon. In addition, the system can be cycled through several times.

Last, to establish if our sensing system could permit H_2S detection in more complex biological media, we tested it in human plasma. Although we were unable to detect a difference in the fluorescence intensity recorded with samples untreated or treated with lead acetate, to trap hydrogen sulfide as insoluble lead sulfide,[30] supplementing the plasma with increasing concentrations of sodium hydrosulfide results in an increase in the fluorescence intensity (Figure 6, left). Moreover, the sensor displays a good stability in the plasma (the fluorescence is unchanged after 10 minutes), does not exhibit any significant reactivity with high concentrations of glutathione (10 mM), and permits real time monitoring of H_2S release by a glutathione-activated donor[31] (Figure 6, right).

Figure 5. Left: selectivity of the fluorescent detection system towards potential interfering biomolecules at physiologically relevant concentrations (5 mM of glutathione, 1 mM for sodium thiosulfate and 100 µM for other substrates). Conditions: 1 µM of fluorophore, 20 µM HbI, in buffer A with 0.9 % DMSO, with (red) or without (black) 5 µM NaSH. Right: reversibility of the detection of hydrogen sulfide. Each cycle consists in i) the addition of 40 µM sodium hydrosulfide and 2) the bubbling of argon for 5 minutes. The reaction was carried out with 1 µM of fluorophore and 12 µM rHbI in buffer A with 0.9 % DMSO. The reaction mixture also contains catalase (500 U) that was found to stabilize the system during the bubbling phase.

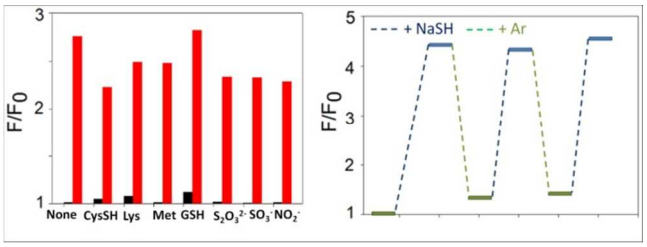
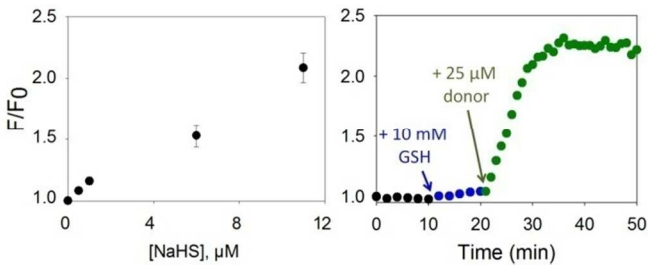


Figure 6. Left: Emission fluorescence ratio recorded after spiking samples of human plasma with sodium hydrosulfide (two samples from different donors were used, with n = 2 for each). Conditions: 1 µM of fluorophore and 12 µM of rHbI, in plasma (with 0.9 % DMSO final). Data are presented as the mean ± S.D. (n=4). Right: Continuous monitoring of the fluorescence intensity under various conditions in plasma (with 0.9 % DMSO final). 0-10 min: sensing system alone (recording every 2 minutes); 10-20 min: addition of 10 mM of GSH at t=10 min (recording every 2 minutes); 20 – 50 min: addition of 25 µM of the donor at t= 20 min (recording every minute).



DISCUSSION

To design an efficient molecular gating system, the choice of both the sensor and the transducer are crucial. Thus, we decided to work with the recombinant hemoglobin rHbI from the clam *Lucina Pectinata* as a sensor. Indeed, rHbI has been reported to be the most avid biological system for hydrogen sulfide and it should accordingly enable low detection limits. In addition, HbI exhibits a strong absorbance band in the Soret region, which is modulated in wavelength and intensity by the coordination of H2S. While the high affinity of rHbI for H2S has been attributed to the combination of a very fast association and of a slow dissociation step, we observed inconsistencies between the dissociation constants determined either under kinetic or thermodynamic conditions. Accordingly, we reinvestigated the kinetics for hydrogen sulfide reactivity with the ferric center of rHbI. Our experiments validated the fast coordination of H2S to rHbI, but they revealed that the dissociation of H2S from rHbI takes place at a faster rate than the one previously reported. This discrepancy most probably lies in the slow recording interval used by Kraus *et al.*[19] (600 s) for their kinetic study. The new half-life (92 s) for the unbinding of H2S allows us to reconcile the kinetic and thermodynamic data, and it is also fully consistent with the reversibility of our sensing system towards H_2S (Figure 5B) which takes place within minutes.

For the transducer, we selected a derivative of the coumarin Pacific Blue™ (PB) based on its photophysical properties. Its combination with rHbI results in significant improvements of our sensing system over those also based on the "absorption into fluorescence" approach to detect gaseous molecules like nitric oxide[18, 32] or dioxygen.[18] First, by choosing a fluorophore with a maximum excitation wavelength corresponding to that of the unliganted sensor rHbI, our system acts as a turn-on sensor, a strategy usually preferred over turn-off systems. Second, a large variation of the fluorescence signal intensity is observed between the off-form of the sensor rHbI and the turned-on form rHbI-H_2S. Indeed, a 17-fold variation in the fluorescence intensity F/F_0 is recorded upon saturation with H_2S in a system containing 20 µM of rHbI and 1 µM PB. For comparison, variations of 1.3- and 7.1-fold were respectively obtained for the detection of nitric oxide with the couples cytochrome c peroxidase / fluoresceine and cobalt-myoglobin / cyan fluorescent protein (CFP). Third, our gated system enables the fast and easy quantification of hydrogen sulfide concentrations down to two hundred nanomolar. While this detection limit is middling compared to chromatographic methods that permit the detection of as low as 1 nM H_2S concentration,[33] our system presents several advantages compared to the later. Due to the extremely fast coordination step of the analyte to rHbI, H_2S detection occurs in real time, a feature so far only proposed by polarographic sensors.[8] In addition, in contrast to classical fluorescent probes which are irreversibly consumed upon reaction with hydrogen sulfide, the coordination of H_2S to rHbI is reversible under our experimental conditions. This allows the reversible detection of H_2S, since no redox reaction is taking place between the analyte and the metal centre. Last, since the active site of rHbI only accommodates small uncharged ligands,[21, 34] the fluorescent detection of H_2S is also selective, and physiological concentrations of other biological thiols, such as glutathione or cysteine, do not interfere with our gated system.

Prominently, we applied our fluorescent sensing system to the quantification of hydrogen sulfide in human plasma. Since the regain of interest for the biological role of H_2S in the late 1990's, plasmatic concentrations ranging from sub-micromolar to hundreds of micromolar have been allegated in the literature,[5] albeit mostly because of erroneous analytical procedures (such as the use of the methylene blue test[6]). On our side, we were unable to unambiguously detect H_2S in unspiked plasma samples with our sensor system. Thus, we did not observe any significant differences between untreated samples and samples treated with lead acetate to remove hydrogen sulfide. However, an immediate increase in fluorescence was recorded upon spiking plasma samples with concentrations as low as 1 µM hydrogen sulfide, indicating that our sensor operates in plasma and that H_2S concentrations in our samples are below the quantification limit. This was confirmed by the monitoring of H_2S release by a gluthathione activated H_2S donor These results are in agreement with a very recent study that uses up to date LC-MS/MS techniques to detect H_2S in biological matrices.[35]

CONCLUSION

Overall, our results confirm the potential of the "absorption into fluorescence" strategy to detect analytes of biological interest. Thus, the combined use of the most avid biosystem for hydrogen sulfide and a rationally selected fluorophore allowed us to design a promising new fluorescence turn-on sensor for hydrogen sulfide. Our gated system detects down to two hundred nanomolar concentration of H_2S, and is selective towards other biological thiols. Significantly, the detection occurs in real time, and reversibly. Although we focused on the application of the sensor in a biological media, we could also envision its use in more complex systems, like micro-fluidic devices to analyze environmental samples. To fully take advantage of our system, the immobilization of the protein on a transparent surface would be a great asset, and we are currently working on this in our group.

ASSOCIATED CONTENT

Supporting Information. Additional Figures mentioned in the main text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors and all authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

† K'_d and k' refers to the pH-dependent equilibrium constant or rate constant, at pH 7.4, with the approximation that [NaSH] = [H_2S].

H_2S: hydrogen sulfide; rHbI: recombinant hemoglobin I from *Lucina Pectinata*; PB: N-[2(2-Hydroxyethoxy)ethyl]-6,8-difluoro-7-hydroxy coumarin-3-carboxamide; dtpa: diethylenetriaminepentaacetic acid; NaSH: sodium hydrosulfide.

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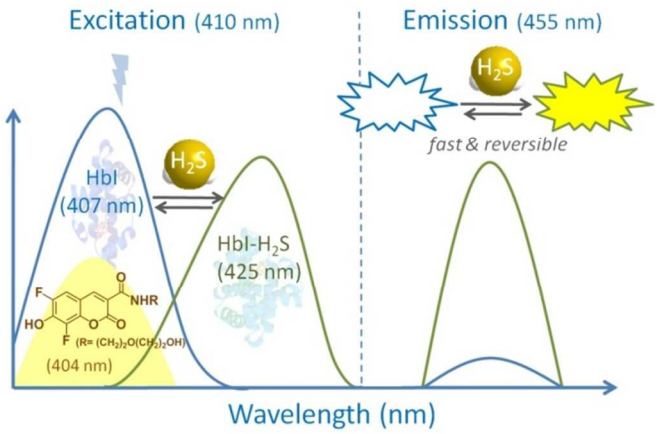
Excitation (410 nm)
Emission (455 nm)
H_2S
fast & reversible
HbI
(407 nm)
H_2S
HbI-H_2S
(425 nm)
F
HO
O
NHR
O
O
F (R= $(CH_2)_2O(CH_2)_2OH$)
(404 nm)
Wavelength (nm)