An off-the-shelf sensor for colourimetric detection of sulfide

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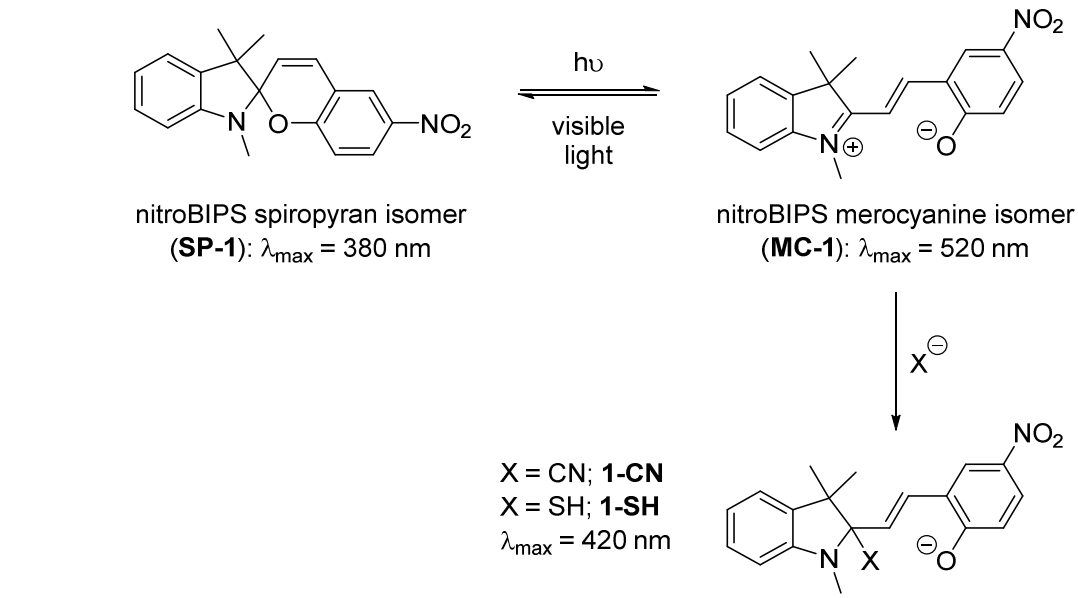
Abstract

The cheap, accessible spiropyran nitroBIPS is a sensitive, selective colourimetric sensor for quantitative determination of sulfide in aqueous media at neutral pH.

Introduction

There is now considerable evidence that supports hydrogen sulfide as being a third physiological gasotransmitter, alongside nitric oxide and carbon monoxide.[1-3] Contemporary research has implicated endogenous hydrogen sulfide release in many important pathologies[4-7] and various hydrogen sulfide donor molecules are undergoing scrutiny as therapeutic agents.[8,9] To support this expanding research field, there has been a tandem development in methods for the detection and quantification of hydrogen sulfide in solution.[10-12] Perhaps the key approach to this goal has been the synthesis of small molecule probes which enable colourimetric and/or fluorimetric measurement of hydrogen sulfide concentration in solution.[11,12] Although the challenges that this presents are manifold, the principal difficulty that an effective probe molecule must overcome is that physiologically relevant hydrogen sulfide concentrations are low (< 1 μM)[13] and occur against a high background concentration of molecules that present similar chemical reactivity (e.g. thiols such as cysteine, homocysteine and glutathione; intracellular glutathione concentration is in the mM range).[14] Several classes of small molecule hydrogen sulfide probe have recently emerged which meet these stringent requirements for selectivity and sensitivity and have been employed with reasonable success in biological media;[11,12] nevertheless, none of these molecules have been adopted widely. We speculate that this is a matter of cost and convenience; there is a need for cheap, "off-the-shelf" probes for the majority of workers, who do not have access to specialist synthetic chemistry facilities. Recent development of a benzofurazan-based sulfide reporter has, in part, addressed this requirement;[15] herein we present a complementary approach.

Spiropyrans (e.g. **SP-1**; Scheme 1) are a class of spiro-fused indolochromene photochromes, commonly deployed in molecular/optical switching and sensing applications.[16-18] The photochromic behaviour displayed by a spiropyran is based upon the light-dependent equilibrium with its zwitterionic merocyanine isomer (e.g. **MC-1**): whereas spiropyrans are usually colourless in solution, merocyanines are fully conjugated, highly coloured and fluorescent (Scheme 1). Spiropyran/merocyanine-based H_2S probes, such as **2** (Figure 1),[19] rely upon nucleophilic attack of HS^- upon the iminium ion present within the merocyanine structure, with the consequent disruption of the extended merocyanine conjugation resulting in reduced visible absorption/fluorescence emission (Scheme 1). As such, spiropyran/merocyanine-based H_2S sensors are often "switch-off" in nature. Such probes discriminate between H_2S and biologically competitive thiols on the basis of acidity: at physiological pH the lower pK_a value of H_2S (~ 7.0) compared with other biologically competitive thiols (e.g. cysteine, glutathione, pK_a > 8.5) ensures a greater degree of dissociation, hence a high concentration of strongly nucleophilic HS^-.



Scheme 1. Spiropyran-merocyanine equilibrium and reaction with cyanide / sulfide

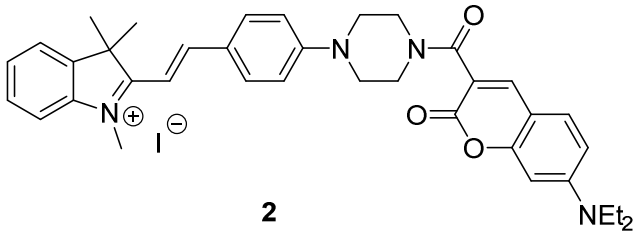


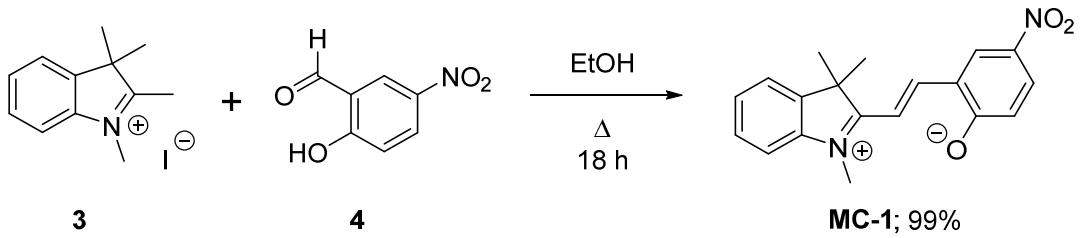
Figure 1. Sulfide probe based upon a merocyanine-coumarin conjugate

1′,3′-Dihydro-1′,3′,3′-trimethyl-6-nitrospiro[2*H*-1-benzopyran-2,2′-(2*H*)-indole] **1**, commonly known as nitroBIPS, is a cheap and readily accessible spiropyran, available both commercially or through facile synthesis.[20] Although the response of nitroBIPS to H_2S has not been investigated, the same molecule has been used as a sensitive, selective probe for anionic cyanide.[21] In this capacity, nitroBIPS could accurately detect [CN^-] to a minimum of 1.7 μM in 1:1 acetonitrile–water and showed a high degree of selectivity for cyanide against a range of potentially competitive anions. It is notable, however, that HS^- was not included and that these experiments were buffered to pH = 9.3 (the authors noted that sensitivity to NC^- was considerably diminished at neutral pH). The product of cyanide addition to nitroBIPS displayed a strong, blue-shifted absorbance distinct from that of the merocyanine; hence the cyanide response could be quantified either by a reduction in merocyanine absorbance and fluorescence (as a switch-off sensor) or by an increase in addition product absorbance (switch-on).

In light of the above studies, nitroBIPS presented an obvious candidate for investigation as an easily-accessible colourimetric /fluorimetric hydrogen sulfide probe. In this work, we assess the performance of nitroBIPS as a colourimetric probe for hydrogen sulfide in aqueous solution at physiological pH.

Results and discussion

NitroBIPS was synthesised *via* the straightforward condensation of tetramethylindolium iodide **3** and nitrosalicylaldehyde **4** in ethanol at reflux (Scheme 2).[20] The merocyanine isomer precipitated from the reaction mixture in excellent yield and high purity and following filtration, no further purification was necessary.



Scheme 2. Synthesis of nitroBIPS

Given the photochromic nature of nitroBIPS and that previous work has identified the merocyanine isomer, and not the spiropyran, as the sole reactive electrophile in detection of cyanide,[21] our initial investigations identified appropriate conditions for use of nitroBIPS as a sulfide sensor by promoting merocyanine formation.

Preparation of a 0.1 mM solution of nitroBIPS in 1:1 acetonitrile–water (PBS 5 mM; pH = 7.4) resulted in a pink solution which displayed a strong absorbance in the visible region (518 nm), corresponding to the merocyanine isomer **MC-1**. This solution decolourised when exposed to standard ambient light for 10 minutes, with the corresponding loss of the 518 nm absorption band attributed to complete isomerisation to spiropyran **SP-1**. If the initially pink solution was kept in darkness, the initial merocyanine concentration remained approximately constant over 30 minutes. Consequently, we adopted dark conditions for the use of nitroBIPS as a sensor molecule in acetonitrile-water. This contrasts somewhat with previous work, where nitroBIPS in 1:1 acetonitrile–water (CHES 100 mM; pH = 9.3) required UV irradiation to ensure the presence of merocyanine; only spiropyran was present in darkness.[21]

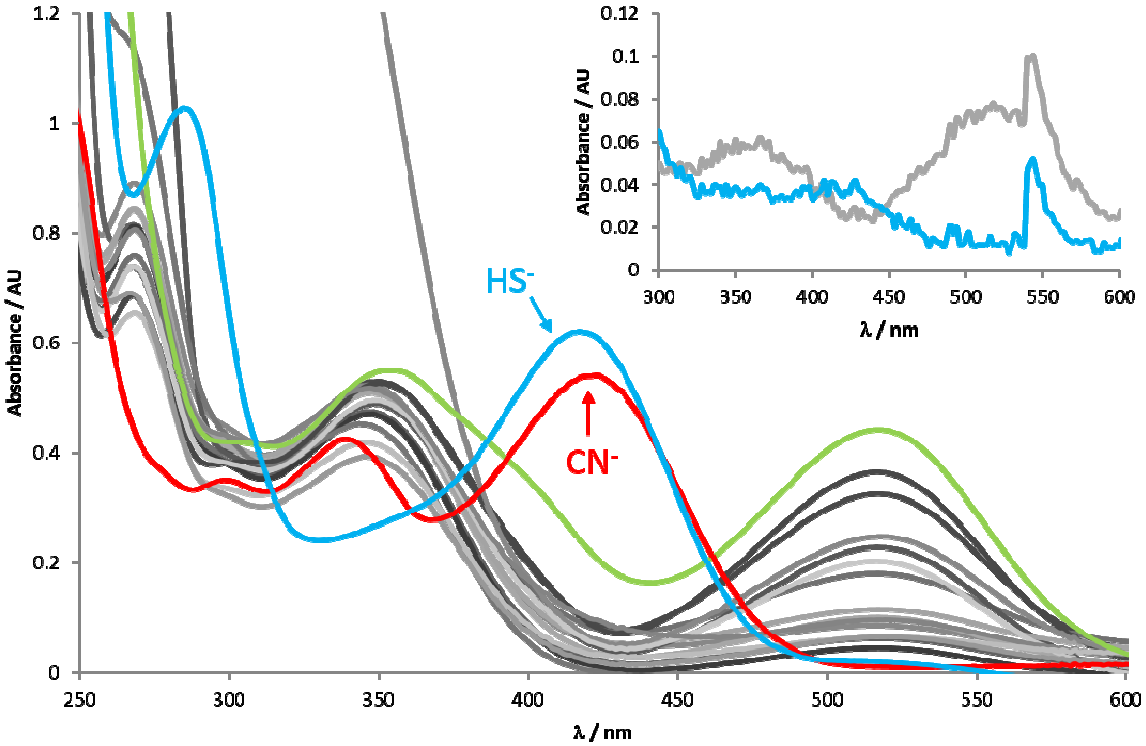


Figure 2. Selectivity and (inset) sensitivity of nitroBIPS as a sulfide sensor. (Main plot) Absorbance spectra of **1** (0.1 mM in 1:1 acetonitrile–water; PBS 5 mM; pH = 7.4) following addition of biologically relevant species (100 µL of 0.1 M solutions in water, 50 equiv.; final concentration = 5 mM) or water control (100 µL) and 30 minutes in darkness. Red line = *n*BuNH$_4$CN; blue line = Na$_2$S·9H$_2$O; green line = Na$_2$SO$_3$; grey lines = no additive, NaCl, KF, KBr, KI, NH$_4$OAc, NaH$_2$PO$_4$·2H$_2$O, Na$_2$SO$_4$·10H$_2$O, NaNO$_3$, NaSCN·H$_2$0, SIN-1·HCl, 3-mercaptopropionic acid, 2-mercaptoethanol, cysteine, glutathione. (Inset) Grey line = absorbance spectra of **1** (5 µM in 1:1 acetonitrile–water; PBS 5 mM; pH = 7.4); blue line = following addition of Na$_2$S·9H$_2$O (25 µL of 20 mM aqueous solution, 50 equiv.; final concentration = 250 µM) and 30 minutes in darkness.

NitroBIPS was then assessed as a sulfide sensor, as follows. Aqueous solutions of sulfide or other potentially competitive species (50 equiv.) were added to individual aliquots containing nitroBIPS (0.1 mM in 1:1 acetonitrile–water; PBS 5 mM; pH = 7.4) then left in darkness for 30 minutes before being analysed by UV-visible spectroscopy. From the results shown in Figure 2, it is apparent that nitroBIPS is unreactive towards most nucleophiles under these conditions: solutions were produced that reflected modulation of the spiropyran-merocyanine equilibrium but did not suggest nucleophilic addition (addition of sodium sulfite resulted in a change in absorbance line shape but without apparent erosion of spiropyran or merocyanine absorbance and without appearance of further visible absorbance peaks). In contrast, in the presence of cyanide, a yellow solution was produced which absorbed strongly at 418 nm and displayed no merocyanine absorbance. A similar result was observed when nitroBIPS was treated with sulfide; however, the resulting solution displayed a further absorbance at 286 nm. On the basis of these results, nitroBIPS can be viewed as a selective sensor for sulfide; competition is only observed from cyanide (with weak interference from sulfite), and sulfide addition can be distinguished from that of cyanide by observation of the λ_{286} absorbance. It is important to note that quantitative sulfide detection *in the presence of cyanide* is not possible under these conditions, which would result in depletion of **MC-1** by cyanide and hence under-reporting of the λ_{286} absorbance. NitroBIPS is reactive towards sulfide but shows no reactivity

towards apparently similar sulfur-based nucleophiles such as mercaptoethanol, mercaptopropionic acid, cysteine and glutathione. This distinction is absolutely crucial because cellular sulfide determination must occur against a high background concentration of cysteine and glutathione.

Quantitative determination of sulfide concentration was achieved by titration of sulfide against nitroBIPS and ratiometric analysis of the characteristic absorbance peaks assigned to **1-SH** (286 nm) and **MC-1** (518 nm). A linear relationship (R^2 = 0.9927) between Na_2S and **1-SH**/**MC-1** was observed, indicating that accurate measurement of sulfide is possible in this range (Figure 3). The lower limit of detection was defined by the average of 5 repetitions of the blank experiment (i.e. in the absence of sulfide) + 3 standard deviations from the mean. In this instance, with [nitroBIPS] = 0.1 mM, this corresponded to a limit of detection of [HS^-] = 10.7 µM or, to provide generality in terms of sensor probe concentration, 1 equivalent of nitroBIPS provides a limit of detection of 0.11 equivalents sulfide. Ultimately, the absolute lower limit of detection will depend upon the lowest possible [nitroBIPS] detectable by the spectrophotometer. Using an entry level spectrophotometer (Jenway 7315), nitroBIPS could be detected to a minimum concentration of 5 µM, at which point sulfide detection remained effective (Fig. 2, inset). Consequently, applying our lower limit of detection to this experiment, we anticipate that statistically relevant detection of [SH^-] = 550 nM is possible.

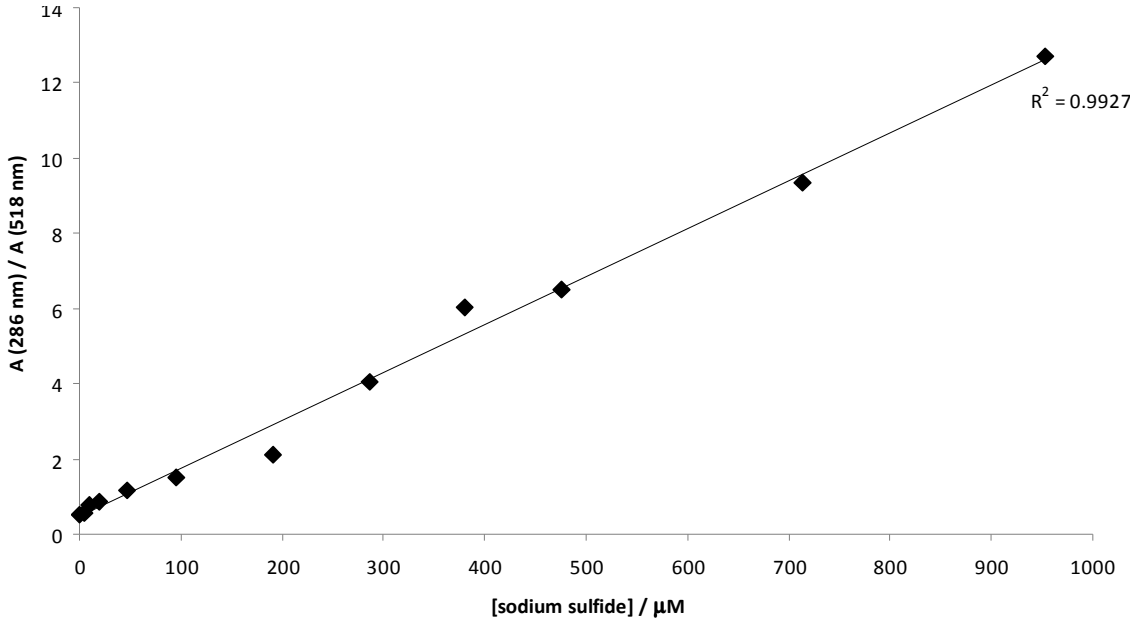


Figure 3. Change in the absorbance ratio (A_{286}/A_{518}) of **1-SH** and **MC-1** with sulfide concentration. Individual samples of **1** (0.1 mM in 1:1 acetonitrile–water; PBS 5 mM; pH = 7.4) and $Na_2S{\cdot}9H_2O$ (0 – 1000 µM) were placed in darkness for 1 hour then analysed by UV-visible spectroscopy.

For a sulfide probe to have biological utility, detection of micromolar sulfide against a background of millimolar glutathione is essential. Consequently, we validated of the utility of nitroBIPS as a sulfide probe by quantification of its response to sulfide in the presence of 5 mM glutathione, using 10 µM nitroBIPS (Figure 4). Initially, we ascertained that nitroBIPS displayed no reactivity towards glutathione (despite the 500-fold excess of the latter), by comparison with a glutathione-free control (Fig. 4; dark and light blue lines). Subsequent introduction of sulfide (25 equivalents, 250 µM) had minimal impact upon the system; a somewhat surprising result given that previously, in the absence of glutathione, similar conditions resulted in formation of **1-SH** (see Figures 2 and 3). At higher sulfide concentrations, reaction with nitroBIPS became effective and gave a linear ratiometric response (A_{420}/A_{518}) to 10 µM increments in sulfide concentration (Fig. 4, inset). We surmise that this

apparent reduction in sulfide sensitivity is actually a reflection of the HS^- available to react with nitroBIPS. We suggest that glutathione has no direct impact upon nitroBIPS but that it modifies the sulfide pool equilibria in such a way as to reduce [HS^-]; perhaps through reaction of persulfide anion with reduced glutathione.[22] We observed a similar, apparently reduced response to sulfide in the presence of cysteine, and reduced sulfide sensitivity in the presence of glutathione and cysteine has been reported with other sulfide probes.[15]

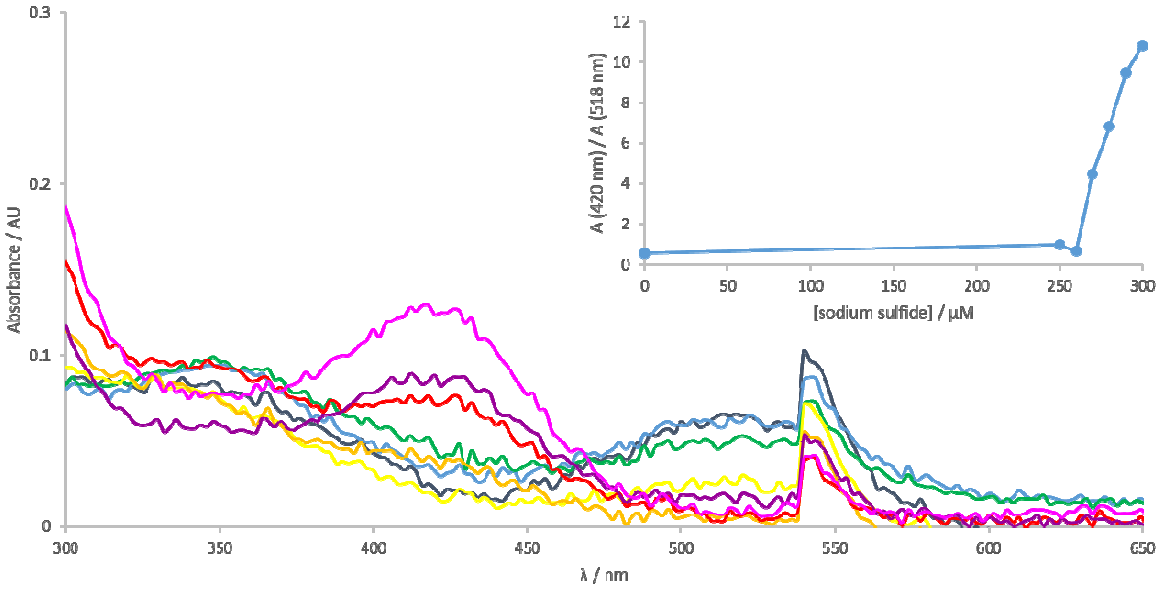


Figure 4. Quantification of [sulfide] using nitroBIPS in the presence of glutathione. (Main plot) Absorbance spectra of **1** (10 µM in 1:1 acetonitrile–water; PBS 5 mM; pH = 7.4): dark blue line = control; all other lines represent samples containing glutathione (5 mM) and $Na_2S{\cdot}9H_2O$ (0 – 300 µM as follows) recorded after 30 minutes in darkness: light blue = 0 µM; green = 250 µM; yellow = 260 µM; orange = 270 µm; red = 280 µm; purple = 290 µM; pink = 300 µM. (Inset) Change in the absorbance ratio (A_{420}/A_{518}) of **1-SH** and **MC-1** with sulfide concentration in the presence of glutathione, extracted from the spectra above.

All analyses to this point were conducted in a mixed solvent system to mitigate against the low solubility of nitroBIPS in water. Given our interest in sulfide detection in biological media, we briefly examined the effectiveness of nitroBIPS as a sulfide sensor in biologically compatible solvents. NitroBIPS is partially soluble in water at 0.1 mM and further dilution does not prevent partial precipitation. Presumably, this reflects the equilibrium between neutral, insoluble *spiro*- and zwitterionic, soluble *mero*-forms. Consequently, equilibrium driven-precipitation of spiropyran will always prevent full solubilisation. Despite this, sufficient merocyanine remains in solution for nitroBIPS to function as a sulfide sensor in water: addition of 50 equivalents of $Na_2S.9H_2O$ to a 0.1 mM suspension of nitroBIPS in water, followed by standing in darkness, resulted in complete conversion of **MC-1** to **1-SH** (Figure 5). As such, nitroBIPS is a useful qualitative sulfide probe in water, but is not suitable for quantitative analysis. On the other hand, nitroBIPS *is* soluble in 1%(v/v) aqueous DMSO at 0.1 mM and can be used to determine sulfide concentration in this medium.

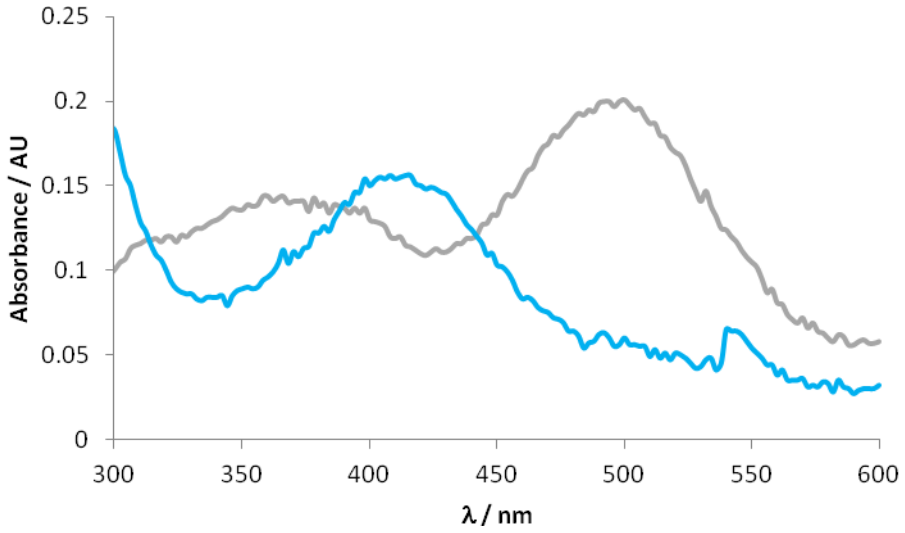


Figure 5. Absorbance spectra of **1** (0.1 mM suspension) in water (PBS 10 mM; pH = 7.4): (a) before addition of Na_2S (grey line); (b) following addition of $Na_2S{\cdot}9H_2O$ (100 µL of 0.1 M solution in water, 50 equiv.) and standing in darkness for 30 minutes (blue line).

Following these initial studies, the interactions of nitroBIPS with cyanide and sulfide were compared in greater depth to identify conditions that would enable quantitative determination of sulfide in the presence of cyanide. At pH = 9.3, cyanide addition to nitroBIPS is light-reversible (hence the sensor can be regenerated with white light)[21] and we observed similar behaviour at pH = 7.4 with both cyanide and sulfide. Solutions of **1-SH** and **1-CN** were prepared by sulfide or cyanide addition, respectively, to solutions of nitroBIPS, then kept in darkness for 30 minutes. These solutions were then irradiated with white light for 30 minutes. Both solutions displayed a degree of photoreversion, and although this effect was more pronounced in the cyanide case (UV-visible spectroscopy showed 60% / 30% decline in 518 nm absorbance for cyanide / sulfide samples, respectively), the limited extent of this differential reactivity is not analytically useful in distinguishing sulfide from cyanide.

Shiraishi and co-workers demonstrated that cyanide addition could only occur *via* the merocyanine isomer of nitroBIPS and therefore employed UV irradiation to ensure a high merocyanine concentration.[21] Conversely, we investigated the addition of cyanide and sulfide to nitroBIPS under conditions in which the merocyanine concentration was minimised, in order to exploit the greater nucleophilicity of sulfide over cyanide. Solutions of nitroBIPS were irradiated with white light for 1 minute to produce solely the spiropyran isomer, before adding cyanide/sulfide and placing in darkness. In both cases, addition of the anion to nitroBIPS occurred slowly; however, whereas cyanide addition was negligible after 2 hours, sulfide addition was significantly more effective (Fig. 6). To validate this encouraging result, the same illumination/addition protocol was followed to give a nitroBIPS solution containing both cyanide and sulfide. Gratifyingly, this displayed near-identical behaviour to that containing sulfide alone, confirming the negligible effect of cyanide under these conditions (Fig. 6, inset). Consequently, although a long (2 h) measurement time is necessary, nitroBIPS *can* function as a sensor for sulfide in the presence of cyanide, provided that the merocyanine isomer is eliminated from the system as a starting position by irradiation with white light: quantitative determination of [SH^-] can be achieved through ratiometric analysis of **1-SH** (286 or 418 nm) and **SP-1** (348 nm), as opposed to **MC-1** as used previously.

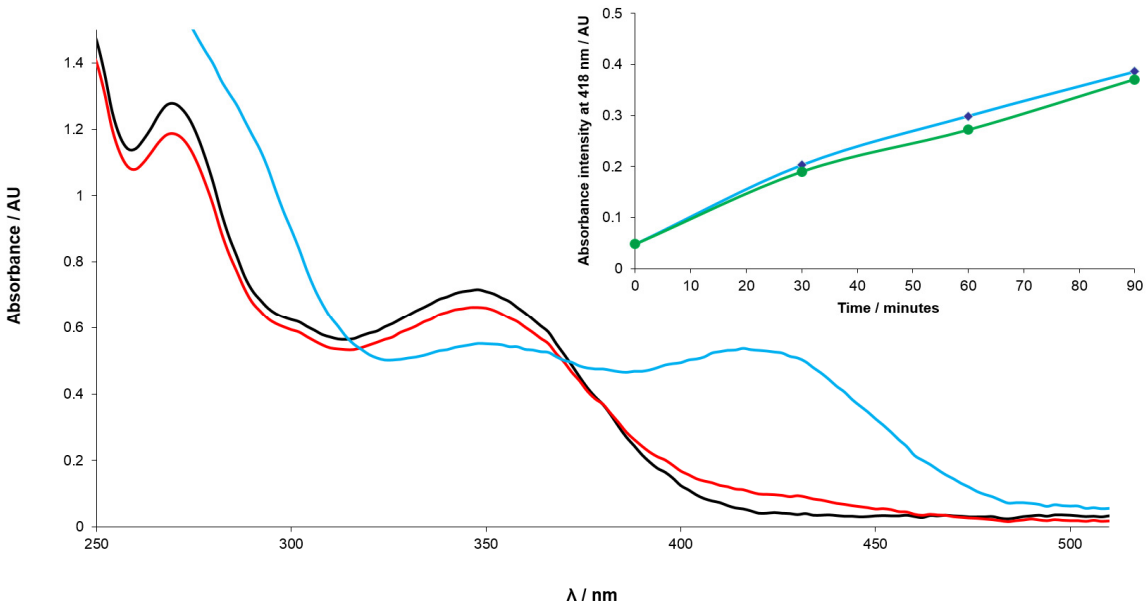


Figure 6. Absorbance spectra of **1** (0.1 mM in 1:1 acetonitrile–water; PBS 5 mM; pH = 7.4) following 1 minute irradiation with white light: (a) before sulfide/cyanide addition (black line); (b) following addition of Bu_4NCN (200 µL of 0.05 M solution in water, 50 equiv.) and standing in darkness for 2 hours (red line); (c) following addition of $Na_2S{\cdot}9H_2O$ (200 µL of 0.05 M solution in water, 50 equiv.) and standing in darkness for 2 hours (blue line). (Inset) Change in absorbance intensity at 418 nm over time (samples placed in darkness): (a) following addition of $Na_2S{\cdot}9H_2O$ (200 µL of 0.05 M solution in water, 50 equiv.) (blue line); (b) following addition of $Na_2S{\cdot}9H_2O$ (100 µL of 0.1 M solution in water, 50 equiv.) and Bu_4NCN (100 µL of 0.1 M solution in water, 50 equiv.) (green line).

Previous work has shown that addition of sulfide[19] or cyanide[21] to merocyanines operates through nucleophilic attack upon the iminium ion to give clean conversion to the corresponding quaternary adduct (Scheme 1). The addition of sulfide to nitroBIPS, although superficially similar, is considerably less straightforward. ^1H and ^{13}C NMR analysis of the sulfide addition product revealed a complex mixture of products with little useful structural information. UV-visible spectroscopy suggests that a major component of addition is similar to the corresponding cyanide adduct **1-CN** (λ_{418}); however, the presence of further absorption peaks corroborates NMR evidence for alternative reaction pathways. In the absence of concrete evidence, it is perhaps unhelpful to speculate upon the outcome of this reaction; however, it is pertinent to note that in addition to direct attack upon the iminium ion, conjugate addition of sulfide to **MC-1** is plausible: conjugate addition of water to nitroBIPS derivatives has been observed over extended reaction times[23] and conjugate addition of sulfur-based nucleophiles to structurally similar cyanine dyes has been reported.[24-26] Furthermore, sulfide is an effective reducing agent (e.g. of nitro groups)[27] and is capable of forming disulfides; consequently, it is unsurprising that nitroBIPS and sulfide react through multiple pathways.

The purpose of this research was to provide an objective analysis of the cheap, well-known spiropyran nitroBIPS as a colourimetric sensor molecule for hydrogen sulfide. In this role, nitroBIPS functions as a ratiometric probe, allowing convenient and rapid determination of sulfide to physiologically relevant nanomolar levels, in aqueous media and at biological pH. NitroBIPS is highly selective for sulfide amongst other biologically relevant species, including thiols such as cysteine and glutathione, and we have demonstrated quantitative determination of micromolar sulfide levels in

the presence of 5 mM glutathione (representative of typical cellular concentrations). NitroBIPS suffers from competition with cyanide; however: (i) cyanide is rarely present in biological samples; (ii) selectivity for sulfide over cyanide can be established through simple modulation of the *spiro-mero* equilibrium. Although nitroBIPS is an effective sulfide probe in aqueous media and can be used in 1% aqueous DMSO, its limited water solubility precludes quantitative sulfide determination in the absence of organic solvents (though qualitative detection is effective under these conditions). Future research will seek to identify fully water soluble analogues and to elucidate the mechanism(s) by which sulfide interacts with nitroBIPS.

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