2,4-dinitrophenyl ether-containing chemodosimeters for the selective and sensitive “in vitro” and “in vivo” detection of hydrogen sulfide

Sameh El Sayed,\textsuperscript{a,b,c} Cristina de la Torre,\textsuperscript{a,b,c} Luis E. Santos-Figueroa,\textsuperscript{a,b,c} Ramón Martínez-Máñez,\textsuperscript{a,b,c,} Félix Sancenón,\textsuperscript{a,b,c} Mar Orzáez,\textsuperscript{d} Ana M. Costero,\textsuperscript{a,e,} Margarita Parra,\textsuperscript{a,e} and Salvador Gil\textsuperscript{a,e}

\textsuperscript{a} Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Unidad Mixta Universitat Politècnica de València – Universitat de València. E-mail: rmaez@qim.upv.es

\textsuperscript{b} Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain.

\textsuperscript{c} CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN).

\textsuperscript{d} Laboratorio de Péptidos y Proteinas, Centro de Investigación Príncipe Felipe, Autopista El Saler, Valencia, Spain.

\textsuperscript{e} Departamento de Química Orgánica, Universitat de València, Dr. Moliner 50, 46100, Burjassot, Valencia, Spain.

Abstract

Four probes (i.e. D1-D4) for the selective and sensitive fluorogenic detection of HS\textsuperscript{−} have been prepared and characterized. HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solutions of D1-D4 are essentially non-fluorescent. Changes in the emission using D1-D4 in the presence of anions (F\textsuperscript{−}, Cl\textsuperscript{−}, Br\textsuperscript{−}, I\textsuperscript{−}, N\textsubscript{3}\textsuperscript{−}, CN\textsuperscript{−}, SCN\textsuperscript{−}, AcO\textsuperscript{−}, CO\textsubscript{3}\textsuperscript{2−}, PO\textsubscript{4}\textsuperscript{2−}, SO\textsubscript{4}\textsuperscript{2−}, HS\textsuperscript{−} and OH\textsuperscript{−}), biothiols (GSH, Cys, Hcy, Me-Cys and lipoic acid), reducing agents (SO\textsubscript{3}\textsuperscript{2−} and S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−}) and oxidants (H\textsubscript{2}O\textsubscript{2}) demonstrated that only HS\textsuperscript{−} is able to induce the appearance of intense emission bands in the 400-520 nm range in the four probes. The selectivity observed was ascribed to a unique hydrogen sulfide-induced hydrolysis of the 2,4-dinitrophenyl ether moiety that yielded the corresponding free highly fluorescent alcohols. The potential detection of intracellular HS\textsuperscript{−} was also studied.
Keywords
Hydrogen sulfide, fluorescence, dinitrophenyl ether, in vivo detection, chemodosimeter

Introduction

Hydrogen sulfide (H\textsubscript{2}S), a colourless and flammable gas with high solubility in water and in organic solvents, is well-known for its unpleasant “rotten eggs” smell. Hydrogen sulfide has been traditionally studied for its toxicity,(1) its presence as a corrosive industrial refuse and emission in the energy industry,(2) as parameter for geological activity,(3) as quality control of foods and as an important compound related with the presence of microbial activity in anaerobic environments.(4) Moreover, this simple molecule has been recently recognized to play a relevant role in living systems. For instance hydrogen sulfide has been documented to be the third gaseous transmitter (the other two being nitric oxide and carbon monoxide).(5-7) The production of H\textsubscript{2}S in mammalian systems has been attributed to at least three endogenous enzymes, i.e. cystathionine b-synthase (CBS), cystathionine g-lyase (CSE), and 3-mercaptopropionate sulfurtransferase (MPST).(8-11) These enzymes use cysteine or cysteine derivatives as substrates and convert them into hydrogen sulfide in different organs and tissues. In addition to these enzymatic pathways, there are also a range of simple chemical events that may liberate hydrogen sulfide from the intracellular pool of “labile” sulfur such as the so-called “sulfane pool” (compounds containing sulfur atoms bound only to other sulfur atoms).(12) The production of endogenous hydrogen sulfide and exogenous administration of hydrogen sulfide has been demonstrated to exert protective effects in many pathologies. For example, hydrogen sulfide has been shown to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure.(13) Hydrogen sulfide has proved also to inhibit leukocyte adherence in mesenteric microcirculation in vascular inflammation in rats, suggesting that hydrogen sulfide is a potent anti-inflammatory molecule.(14) Additionally, it has become evident that hydrogen sulfide is a potent antioxidant and, under chronic conditions, can up-regulate antioxidant defense.(15)

However hydrogen sulfide exact mechanism of action and production in living systems is still under active investigation. For example it is known that hydrogen sulfide can react readily with methemoglobin to form sulfhemoglobin, which act as the metabolic sink for H\textsubscript{2}S. As a potential reductant, hydrogen sulfide is likely to be consumed by endogenous oxidant species such as hydrogen peroxide, superoxide,
peroxynitrite, etc. This process is significant and it provides a mechanism by which the concentration of hydrogen sulfide changes. Besides, abnormal levels of hydrogen sulfide have been associated with various diseases such as chronic kidney disease, liver cirrhosis,(16) Alzheimer,(17) and Down syndrome.(18)

Due to the important roles that hydrogen sulfide plays in environmental and in biological processes the development of selective detection systems for this compound has recently become a focus of importance. In particular it was a major challenge to clarify the complex contributions of H₂S in certain diseases by finding methods for selective tracking of this small molecule within living systems. Current techniques for hydrogen sulfide detection, such as colorimetry,(19-21) electrochemical assays,(22) gas chromatography(23) and metal-induced sulfide precipitation(24) show some shortcomings typically involving destruction of the sample and/or poor selectivity.

In addition to the above mentioned classical methodologies, fluorescence is an attractive highly sensitive technique for studying biomolecule distribution in living cells. **For instance, very recently, fluorescent probes for monitoring redox cycles in vivo have been described.** (25) In this area, several fluorescent probes, designed taking into account supramolecular chemistry concepts, for the selective recognition of H₂S or HS⁻ have been described. (26) Most of the fluorescent probes reported are based of the hydrogen sulfide-induced reduction of azides or nitro moieties, electronically connected with a fluorophore, to amines. (27-36) This reduction induced perturbations in the electronic structure of the signaling unit that is reflected in emission changes. Another important group of probes are constructed under a displacement paradigm and are based on non-fluorescent Cu(II) complexes. (37-46) In these systems the hydrogen sulfide-induced demetallation of the probes induced marked emission enhancements. Other sulfide-selective fluorescent probes used Michael type addition reactions coupled with emission changes. (47-50) Also, cyclization processes that yielded fluorescent compounds have recently been used for the design of fluorescent probes for sulfide anion. (51-53) In other examples, hydrolysis reactions coupled with emission changes have been also used for the preparation of other hydrogen sulfide sensitive probes. (54-56) **Finally, selenium containing BOPIPY dyes have also been used for the reversible detection of sulfide in solution and in living cells.** (57,58) Among probes that used hydrolysis reactions, as far as we know only three probes containing 2,4-dinitrophenyl ethers have been described and used for the selective and sensitive recognition of HS⁻.
anion. In particular, Lin and co-workers prepared a NIR dye functionalized with a 2,4-dinitrophenyl ether moiety.\(^{(59)}\) PBS (pH 7.0)-ethanol 9:1 v/v solutions containing CTAB and this probe were non-fluorescent whereas addition of increasing quantities of HS\(^{-}\) anion induced a progressive appearance of an emission band. This emission enhancement was ascribed to a thiolysis of the 2,4-dinitrophenyl ether moiety. The same sensing mechanism was used by Spring and co-workers that prepared a 1,8-naphthalimide fluorophore equipped with a 2,4-dinitrophenyl ether group.\(^{(60)}\) Finally, Liu and Feng prepared an ESIPT (excited state intramolecular proton transfer) probe functionalized with a 2,4-dinitrophenyl ether moiety for the rapid chromo-fluorogenic recognition of hydrogen sulfide.\(^{(61)}\) The above described facts demonstrate that the design of new fluorogenic probes for hydrogen sulfide is a timely field of interest. Such simple systems may be used to control H\(_2\)S concentrations in target industrial processes, for in situ environmental, geological monitoring and detection of hydrogen sulfide levels in living systems. Nevertheless, in spite of these possible applications, there are still relatively few examples of optical probes for the selective and sensitive detection of hydrogen sulfide (\textit{vide ante}).

Bearing in mind the above mentioned concepts and following our interest in the development of new approaches for optical sensing,\(^{(62)}\) we show herein a family of chemodosimeters (D\textsubscript{1}-D\textsubscript{4}) composed by selected fluorophores functionalized with 2,4-dinitrophenyl ether moieties for the detection of hydrogen sulfide. The synthesis and sensing features of probe D\textsubscript{3} were recently published by us in a short communication.\(^{(63)}\) These dosimeters were easily synthesized through nucleophilic aromatic substitution reactions with 1-fluro-2,4-dintrobenzene with different fluorophores. The four probes displayed sensing features in buffered aqueous solution containing 1% of DMSO and gave marked emission enhancements only in the presence of HS\(^{-}\) anion, whereas other bio-thiols were unable to induce emission changes. The probes showed excellent sensitivities, with limits of the detection in the 0.05-0.30 µM range, and some of the chemosensors were used for the fluorescence detection of both exogenous and endogenous HS\(^{-}\) in living cells.

**Results and discussion**

**Synthesis and characterizations of the probes:** The path of synthesis and the structures of probes D\textsubscript{1}-D\textsubscript{4} are shown in Scheme 1. D\textsubscript{1}-D\textsubscript{4} are 2,4-dinitrophenyl ether derivatives which are easily obtained in good yield (65 to 88%) through an aromatic
nucleophilic substitution reaction between 1-fluoro-2,4-dinitrobenzene (1) and alcohols 1c, 2a, 3a and 4b. Compounds 2a and 3a are commercially available. Alcohol 1c was prepared through the base-catalysed condensation between 4-hydroxybenzaldehyde (1a) and 4-picoline (1b), whereas alcohol 4b was obtained through methylation of fluorescein (4a) with methyl iodide.

Scheme 1. Synthesis and chemical structure of chemodosimeters D1-D4.

All new probes were characterized by 1H, 13C-NMR and HRMS. At this respect, the more indicative signals in the 1H-MNR spectra of dosimeter D1 in DMSO-D6 were the doublets centred at 7.81 and 7.32 ppm attributed to the protons of the trans double bond. Moreover the protons of the 2,4-dinitrophenyl ether moiety appeared at 8.91 (1H, d), 8.47 (1H, dd) and 7.57 (1H, d) ppm. On changing to chemodosimeter D2, the most characteristic signals in the 1H-NMR (CDCl3) spectrum were also those of the 2,4-dinitrophenyl ether moiety centred at 8.97, 8.41 and 7.39 ppm. Nearly the same pattern was observed for D3 in which the dinitrophenyl ether protons were located at 8.93 (1H, d), 8.18 (1H, dd) and 7.83 (1H, d) ppm. Dealing with fluorescein derivative D4, the signals ascribed to the dinitrophenyl ether appeared at nearly the same chemical shift.
than those observed for D1-D3, whereas the methoxy moiety appeared as singlet at 3.76 ppm (in DMSO-D$_6$).

**Sensing features of the probes:** The four synthesized probes contain a chromo-fluorogenic subunit functionalized with a sulfide-sensitive 2,4-dinitrophenyl ether moiety. It is well known that the dinitrophenyl group has been often used for tyrosine protection in peptide synthesis.(64) The removal of this protective group is carried out using thiols under basic conditions. In our prepared probes the dinitrophenyl ether was electronically coupled with the fluorophore and the rupture of the ether bond was expected to induce changes in the emission spectra of the fluorogenic subunit. This simple sensing mechanism was used, very recently, in the preparation of selective and sensitive chemodosimeters for HS$^-$ anion in water and in cellular media (vide ante).(59-61) The high selectivity obtained for HS$^-$ versus thiols (vide infra) relies on the fact that H$_2$S is a small molecule with a pK$_a$ of 6.9, while typical cellular free thiols (i.e. GSH and Cys) have pK$_a$ values of ca. 8.5.(65) Taking into account these differences, at physiological pH, the thiolysis of 2,4-dinitrophenyl ethers is chemoselective for HS$^-$ over GSH, Cys and other biologically-relevant thiols.

**Figure 1.** Emission intensity of D1 (at 515 nm upon excitation at 390 nm), D2 (at 415 nm upon excitation at 350 nm), D3 (at 516 nm upon excitation at 450 nm) and D4 (at 515 nm upon excitation at 450 nm) dosimeters (5 µM) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solutions upon addition of 10 equivalents of anions, bio-thiols, reducing agents and oxidants (after 60, 70,
30 and 50 minutes of the addition for D1, D2, D3 and D4 respectively. The temperature was set at 25°C.

In a first step we tested the fluorescent response of the probes D1-D4 (5 µM) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of an excess (10 equivalents) of selected anions (F⁻, Cl⁻, Br⁻, I⁻, N₃⁻, CN⁻, SCN⁻, AcO⁻, CO₃²⁻, PO₄³⁻, SO₄²⁻, HS⁻ and OH⁻), biothiols (GSH, Cys, Hcy, Me-Cys and lipoic acid), reducing agents (SO₃²⁻ and S₂O₅²⁻) and oxidants (H₂O₂). As it can be seen in Figure 1 the four dosimeters were practically non-fluorescent, however addition of HS⁻ induced a very remarkable fluorescent “turn-on” response, whereas addition of the other chemicals tested induced negligible changes in the emission behaviour. Besides, the fluorescent behavior of the four probes in the presence of GSH 6 mM (GSH concentrations in cells are in the 1-10 mM range) was also tested. As could be seen in Figure 2 GSH was unable to induce a remarkable fluorescent enhancement of the four probes even at mM concentrations.

**Figure 2.** Emission intensity of D1 (at 515 nm upon excitation at 390 nm), D2 (at 415 nm upon excitation at 350 nm), D3 (at 516 nm upon excitation at 450 nm) and D4 (at 515 nm upon excitation at 450 nm) dosimeters (5 µM) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solutions alone (white bars) and upon addition of 50 µM GSH (light grey bars), 6 mM GSH (dark grey bars) and 50 mM HS⁻ (black bars). The temperature was set at 25°C.
Figure 3. Fluorescence enhancement of dosimeter D1 (5 µM) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of HS⁻ anion (from 0 to 10 equivalents) after 60 minutes of the addition (λ<sub>ex</sub> = 390 nm). The temperature was set at 25°C.

More in detail, addition of 10 equivalents of HS⁻ anion to HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution of dosimeter D1 induced the appearance of an emission band at 515 nm (λ<sub>ex</sub> = 390 nm) that reached its maximum intensity after 60 minutes (15-fold enhancement) (see Figure 3). For D2, addition of HS⁻ induced a 46-fold enhancement of the emission intensity at 415 nm (λ<sub>ex</sub> = 350 nm) after 70 minutes (Figure 4).

Figure 4. Fluorescence enhancement of dosimeter D2 (5 µM) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of HS⁻ anion (from 0 to 10 equivalents) after 70 minutes of the addition (λ<sub>ex</sub> = 350 nm). The temperature was set at 25°C.
Figure 5. Fluorescence enhancement of dosimeter D4 (5 µM) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of HS⁻ anion (from 0 to 10 equivalents) after 30 minutes of the addition (λ_{ex} = 450 nm). The temperature was set at 25°C.

Figure 6. Fluorescence enhancement of dosimeter D3 (5 µM) in HEPES (10 mM, pH 7.4)-DMSO 99:1 v/v solution upon addition of increasing quantities of HS⁻ anion (from 0 to 10 equivalents) after 50 minutes of the addition (λ_{ex} = 450 nm). The temperature was set at 25°C.

Nearly the same enhancement (32-fold) in the intensity at 515 nm (λ_{ex} = 450 nm) was obtained upon addition of HS⁻ anion to probe D4 (Figure 5). In this case the response was faster (when compared with D1 and D2) as the maximum enhancement was observed after 30 minutes upon HS⁻ addition. Besides, dosimeter D3 gives the larger response, in this case the addition of HS⁻ anion induced a remarkable 344-fold enhancement in the emission band at 516 nm (λ_{ex} = 450 nm) after 50 minutes (see
Figure 6). The subtle differences in response time and in emission enhancement upon addition of HS\(^-\) anion to the four dosimeters could be ascribed to the different electronic properties of the probes although in all cases a clear highly selective *turn-on* response was observed.

After assessing the highly selective turn-on response of D1-D4 to hydrogen sulfide, the sensitivity of the probes was studied from titration profiles with HS\(^-\) obtained in HEPES-DMSO 99:1 v/v solutions. From these studies a limit of detection (LOD) of 0.20 µM for HS\(^-\) was determined for D1. Nearly the same response and sensitivity was obtained with probe D2 (LOD = 0.29 µM). A more sensitive response was found on changing to dosimeters D3 and D4. In these cases the titration profiles obtained allowed to calculate LOD of 0.06 and 0.05 µM for D3 and D4, respectively. In all cases the probes displayed LODs below the hydrogen sulfide concentration required to elicit physiological responses (10-1000 µM).(66)

The selective emission enhancements observed for D1-D4 in the presence of HS\(^-\) is ascribed in all cases to an HS\(^-\)-induced hydrolysis of the 2,4-dinitrophenyl ether groups that results in the release of the highly fluorescent alcohols 1c, 2a, 3a and 4b. In order to corroborate this hypothesis probes D1-D4 were dissolved in ethanol and an excess of Na\(_2\)S was added. The reactions were stirred at room temperature for 3 h and then the solvent was eliminated in a rotary evaporator. The crudes obtained were purified by silica column chromatography and \(^1\)H and \(^{13}\)C confirmed the presence of the alcohols 1c, 2a, 3a and 4b (for D1, D2, D3 and D4 respectively) as main products of the reaction.

The sensing features of D1-D4 probes were quite similar to those reported for analogous fluorophores functionalized with 2,4-dinitrophenylethers (see Table 1). In particular, reaction times when using hydrolysis of 2,4-dinitrophenylether as sensing mechanism are in the range of minutes whereas the detection limits are in the 0.05-0.29 µM interval. Table 1 also shows the typical sensing parameters for probes for hydrogen sulfide when using different sensing mechanisms. From the data in the table it is apparent that the best response, in terms of reaction time, are obtained with probes in which the sensing mechanism is related with demetallation of Cu(II) complexes (from few seconds to minutes). As seen also in Table 1, lower limits of detection for hydrogen sulfide are obtained when demetallation processes and reduction reactions are used as transduction mechanisms.
Table 1. Mechanism used in the development of fluorogenic probes for hydrogen sulfide.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Time</th>
<th>Limit of detection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrolysis of 2,4-dinitrophenylether (D1-D4)</td>
<td>30-70 minutes</td>
<td>0.05-0.29 µM</td>
<td>this paper</td>
</tr>
<tr>
<td>hydrolysis of 2,4-dinitrophenylether</td>
<td>5-20 minutes</td>
<td>0.05-0.48 µM</td>
<td>59-61</td>
</tr>
<tr>
<td>demetallation of Cu(II) complexes</td>
<td>few seconds-5 minutes</td>
<td>0.01-16 µM</td>
<td>37-46</td>
</tr>
<tr>
<td>Michael addition reactions</td>
<td>2-60 minutes</td>
<td>0.12-1 µM</td>
<td>47-50</td>
</tr>
<tr>
<td>other hydrolysis reactions</td>
<td>30-60 minutes</td>
<td>0.05-9 µM</td>
<td>54-56</td>
</tr>
<tr>
<td>reduction reactions</td>
<td>3-120 minutes</td>
<td>0.01-5 µM</td>
<td>27-36</td>
</tr>
<tr>
<td>oxidation of Se-containing fluorophores</td>
<td>5 minutes</td>
<td>not reported</td>
<td>57,58</td>
</tr>
<tr>
<td>cyclization reactions</td>
<td>2-50 minutes</td>
<td>0.1-100 µM</td>
<td>51-53</td>
</tr>
</tbody>
</table>

Figure 7. Cell viability assays. Hela cells were treated with dosimeters D1 (black), D2 (dark grey), D3 (grey) and D4 (white) at concentrations of 5,10,20,50 µM during 24h.

Cell viability assays: The selective emission enhancement of D1-D4 in the presence of HS\(^-\) and the poor response observed upon the addition of bio-thiols (GSH, Cys and Hcy) strongly suggest that these probes could be used for HS\(^-\) imaging in living cells. Based on these observations, the cytotoxicity of D1-D4 was first evaluated. For this purpose HeLa cells were treated with probes D1-D4 at different concentrations (5, 10, 20 and 50 µM) over a 24-hour period and cell viability was determined by using a WST-1 assay. The obtained results are shown in Figure 7. As seen, the four dosimeters were essentially non-toxic in the range of concentrations tested.
Detection of HS⁻ in HeLa cells: Once assessed the biocompatibility of the prepared probes, and with the aim to test the dosimeters in highly competitive environments, we prospectively used probes D1-D4 for the fluorescence imaging of sulfide in living cells. Of the four probes tested, only D1 and D3 gave remarkable results due to the low solubility of D2 and D4 at the concentrations required in the confocal microscopy studies.

Figure 8. Detection of HS⁻ levels in living cells using dosimeter D1. (a, e) Transmitted light and fluorescence images of untreated HeLa cells. (b, f) Hela cells incubated with D1 for 90 minutes. (c, g) HeLa cells incubated with D1 for 30 minutes at 37 °C and then with 200 µM Na₂S for 60 minutes. (d, h) Hela cells incubated during 30 min with D1 and 500 µM Na₂S for other 60 minutes. (i) Average I/I₀ intensity ratios after addition of 200 and 500 µM of Na₂S in PBS buffer. The excitation and emission wavelength were 450-470 nm and 510-530 nm. Representative fluorescence images from replicate experiments (n = 3) are shown. Error bars are (SD).

In a typical experiment, HeLa cells were incubated in DMEM supplemented with 10% fetal bovine serum. To conduct fluorescence microscopy studies, HeLa cells were seeded in 24 mm glass coverslips in 6-well plates and were allowed to settle for 24 h. Cells were treated with the probes in PBS-DMSO 99:1 v/v at final concentrations of
10 and 30 μM, for D1 and D3 respectively. After 30 minutes, the medium was removed and solutions of different concentrations of NaHS in PBS were added (0, 100, 200 and 500 μM) and cells were incubated for another 10-minute period.

**Figure 9.** Detection of HS\(^{-}\) levels in living cells using probe D3. HeLa cells were incubated with D3 (30 μM) for 30 min at 37°C in DMEM. Transmitted light and fluorescence images were captured for (a) Hela cells, (b) Hela cells incubated with D3, and Hela cells incubated with D3 in the presence of HS\(^{-}\) at concentrations of (c) 200 μM and (d) 500 μM. The range of excitation was 330-380 nm and emission was monitored for wavelengths exceeding 420 nm.

The results are shown in Figures 8 (for D1) and 9 (for D3). The control experiment (HeLa cells without dosimeters) and the cells incubated with D1 and D3 showed no fluorescence, whereas a clear and marked enhancement in intracellular emission was found in the HS\(^{-}\)-treated cells. The emission enhancement observed was clearly dependent of the amount of NaHS added. The enhancement in the intracellular fluorescence intensity with D1 was quantified by a standard image analysis and the results are shown in Figure 8i. A similar response (not shown) was observed for D3.

**Conclusions**

In summary, here we reported the synthesis and sensing features of four compounds (D1-D4) as fluorescent turn-on probes for hydrogen sulfide. These chemodosimeters were based in a different fluorophores (i.e. styryl pyridine, naphthalene, quinoline and fluoresceine for D1, D2, D3 and D4, respectively) functionalized with a 2,4-dinitrophenyl ether moiety. This chemical modification made the final probes poorly emissive. Probes D1-D4 were easy to prepare and were able to selectively detect the HS\(^{-}\) anion in HEPES(10 mM, pH 7.4)-DMSO 99:1 v/v solutions via a remarkable turn-
on emission. The emission enhancements observed were due to a selective sulfide-induced hydrolysis of the 2,4-dinitrophenyl ether moiety that released the free fluorophores. D1-D4 can selectively and sensitively detected HS\(^-\) anion in water over other anions, biothiols and common oxidants. The titration profiles obtained upon addition of increasing quantities of HS\(^-\) anion to aqueous solutions of the four dosimeters allowed us to determine LODs of 0.20, 0.29, 0.06 and 0.05 µM for D1, D2, D3 and D4 respectively. Besides the four dosimeters showed remarkably low sensitivities below the hydrogen sulfide concentration required to elicit physiological responses. From cell viability assays it was found that the four probes were essentially non-toxic. Moreover, real-time fluorescence imaging measurements confirmed that probes D1 and D3 can be used to detect intracellular HS\(^-\) at micromolar concentrations.

**Experimental section**

**Materials and methods:** UV-visible spectra were recorded with a JASCO V-650 Spectrophotometer. Fluorescence measurements were carried out in a JASCO FP-8500 Spectrophotometer. \(^1\)H and \(^{13}\)C-NMR spectra were acquired in a BRUKER ADVANCE III (400 MHZ), where mass spectra were carried out in a TRIPLETOF T5600 (ABSciex, USA) spectrometer. The chemicals 4-picoline, 2-hydroxynaphthalene, 8-hydroxyquinoline, fluorescein, iodomethane, 1-fluoro-2,4-dinitrobenzene, 4-hydroxybenzaldehyde (98%), acetic anhydride (98%), hexadecyltrimethylammonium bromide (CTABr) and sodium bicarbonate were purchased from Sigma-Aldrich. Triethylamine (98%) was purchased from J. T. Baker. Analytical-grade solvents, potassium hydroxide, sodium sulfate and hydrochloric acid (37%) were purchased from Scharlau (Barcelona, Spain).

**Synthesis of 4-((E)-2-(pyridin-4-yl)vinyl)phenol (1c):** 4-hydroxybenzaldehyde (1a, 500 mg, 4.1 mmol) and 4-picoline (1b, 0.4 mL, 4.1 mmol) were refluxed in acetic anhydride (5 mL) for 24 h. After cooling to room temperature a precipitate appeared. This was collected and recrystallized from ethanol. Then, the solid was dissolved in ethanol-water (20 mL, 1:1 v/v) containing potassium hydroxide (400 mg, 7.1 mmol) and refluxed for 6 h. Afterwards, the crude reaction was neutralized with diluted hydrochloric acid. The product 1c precipitated and was isolated as a yellow solid after filtration (749 mg, 3.8 mmol, 85% yield).
\(^1\)H NMR (400 MHz, DMSO) \(\delta\) 12.10 (s, 1H), 10.79 (d, \(J = 5.6\) Hz, 2H), 9.75 (dd, \(J = 21.4, 11.6\) Hz, 5H), 9.29 (d, \(J = 16.4\) Hz, 1H), 9.10 (d, \(J = 8.5\) Hz, 2H).

**Synthesis of 4-(4-(2,4-dinitrophenoxystyryl)pyridine (D1):** 1c (200 mg, 1.17 mmol) was dissolved in acetone (5 mL) and then triethyl amine (0.5 ml, 3.6 mmol) was added. After that, 1-fluoro-2,4-dinitrobenzene (217.7 mg, 1.17 mmol) dissolved in acetone (5 mL) was added to the crude reaction and the mixture refluxed for 30 min. Then, the acetone was evaporated and the residue taken up in 5% hydrochloric acid (10 mL). A precipitate appeared that was filtered, washed with water and then suspended in 5% sodium hydroxide (15 mL) and stirred for 15 min. After that, the solid was filtered, washed with water and purified by silica column chromatography using hexane-acetone 1:1 v/v as eluent. Dosimeter D1 was isolated as yellow solid (310 mg, 0.85 mmol, 74% yield).

\(^1\)H NMR (400 MHz, DMSO) \(\delta\) 8.91 (d, \(J = 2.8\) Hz, 1H), 8.56 (d, \(J = 6.0\) Hz, 2H), 8.47 (dd, \(J = 9.3, 2.8\) Hz, 1H), 7.81 (d, \(J = 8.7\) Hz, 2H), 7.57 (dd, \(J = 4.9, 3.4\) Hz, 3H), 7.34 – 7.25 (m, 4H).

\(^13\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 154.7, 153.9, 150.1, 144.2, 141.6, 139.6, 134.3, 131.8, 129.7, 129.4, 126.6, 122.0, 121.0, 120.7, 119.7.

HRMS-EI m/z: calcd for C\(_{19}\)H\(_{13}\)N\(_3\)O\(_5\) 363.0855; found: 364.0944 (M+H\(^+\)).

**Synthesis of 2-(2,4-dinitrophenoxynaphthalene (D2):** Dosimeter D2 was prepared by the same experimental procedure used for D1. The final product was isolated as pale yellow solid (725 mg, 2.33 mmol, 88% yield).

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.97 (d, \(J = 2.7\) Hz, 1H), 8.41 (dd, \(J = 9.3, 2.7\) Hz, 1H), 8.09 (d, \(J = 8.9\) Hz, 1H), 8.04 – 8.00 (m, 1H), 7.96 – 7.92 (m, 1H), 7.67 (ddd, \(J = 9.4, 4.2, 2.3\) Hz, 3H), 7.40 – 7.37 (m, 1H), 7.18 (s, 1H).

\(^13\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 156.3, 151.2, 141.6, 139.6, 134.2, 131.7, 131.2, 128.9, 128.1, 127.6, 126.5, 122.2, 119.7, 118.8, 117.6.

HRMS-EI m/z: calcd for C\(_{16}\)H\(_{10}\)N\(_2\)O\(_5\) 310.0590; found: 310.0817 (M\(^+\)).

**Synthesis of 8-(2,4-dinitrohydroxy)quinoline (D3):** Dosimeter D3 was prepared by the same experimental procedure used for D1. The final product was isolated as pale yellow solid (703 mg, 2.25 mmol, 85% yield).
$^1$H NMR (400 MHz, CDCl$_3$) δ 8.93 (d, $J = 2.8$ Hz, 1H), 8.79 (dd, $J = 4.2$, 1.7 Hz, 1H), 8.25 (dd, $J = 8.4$, 1.7 Hz, 1H), 8.18 (dd, $J = 9.3$, 2.8 Hz, 1H), 7.83 (dd, $J = 7.9$, 1.7 Hz, 1H), 7.65 – 7.56 (m, 2H), 7.47 (dd, $J = 8.4$, 4.2 Hz, 1H), 6.79 (d, $J = 9.3$ Hz, 1H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 157.4, 151.0, 149.4, 141.4, 140.6, 138.9, 136.4, 130.3, 128.7, 126.8, 122.4, 120.9.

HRMS-EI m/z: calcd for C$_{15}$H$_{10}$N$_3$O$_5$ 311.0542; found: 312.0628 (M+H$^+$).

**Synthesis of 3-hydroxy-6-methoxy-3H-spiro[isobenzofuran-1,9-xanthen]-3-one (4b):** Fluorescein (4a, 2 g, 6.18 mmol) was suspended in methanol (50 mL) and NaOH (160 mg, 4 mmol) was then added. The final mixture was stirred until complete dissolution of fluorescein. After evaporation of methanol, the disodium salt (2.0 g, 5.32 mmol) was suspended in DMF (50 mL) containing methyl iodide (332 µL, 5.32 mmol) and the reaction was then stirred at room temperature for 48 h. After this time, the crude was diluited with 5% NaHCO$_3$ (50 mL) and extracted with diethyl ether (2 x 25 mL). The organic phase was washed with brine (20 mL), dried over Na$_2$SO$_4$, and evaporated to give an orange precipitate. This solid was dissolved in methanol (30 mL), 2M NaOH (10 mL) was added and the mixture stirred at room temperature for 2 h. After evaporation of methanol, the crude was diluted with water (20 mL) and impurities extracted with diethyl ether (2 x 10 mL). The aqueous phase was acidified (pH 2.0) by the addition of concentrated HCl and then extracted with diethyl ether (3 x 15 mL), washed with brine (25 mL) and dried over Na$_2$SO$_4$. After evaporation of the diethyl ether a brown solid was obtained. The final product was purified by silica column chromatography using diethyl ether-hexane 2:1 v/v as eluent. Compound 4b was isolated as brown solid (831.2 mg, 2.4 mmol, 45% yield).

**Synthesis of 3-(2,4-dinitrophenoxo)-6-methoxy-3H-spiro[isobenzofuran-1,9-xanthen]-3-one (D4):** Dosimeter D4 was prepared by the same experimental procedure used for D1. The final product was isolated as brown solid (162 mg, 0.315 mmol, 65 % yield).

$^1$H NMR (400 MHz, DMSO) δ 8.84 (d, $J = 2.8$ Hz, 1H), 8.58 (d, $J = 3.1$ Hz, 1H), 8.45 (dd, $J = 9.2$, 2.6 Hz, 1H), 8.01 (d, $J = 7.6$ Hz, 1H), 7.90 – 7.77 (m, 1H), 7.73 (t, $J = 7.3$ Hz, 1H), 7.37 (d, $J = 9.2$ Hz, 1H), 7.22 (dd, $J = 9.8$, 5.1 Hz, 1H), 6.92 (ddd, $J = 20.1$, 13.2, 5.6 Hz, 1H), 6.74 (dd, $J = 8.9$, 2.5 Hz, 1H), 6.67 (d, $J = 8.8$ Hz, 1H), 6.45 (d, $J = 9.7$ Hz, 1H), 3.76 (s, 1H).
\[^{13}C\] NMR (101 MHz, DMSO) \( \delta \) 158.3, 155.0, 144.7, 141.2, 140.9, 139.1, 138.7, 138.5, 136.6, 129.7, 127.9, 126.6, 126.0, 125.8, 125.6, 124.8, 123.0, 121.7, 120.3, 117.8, 114.8, 115.8, 113.0, 110.1, 107.8, 56.1, 18.6.

HRMS-EI m/z: calcd for C\(_{27}\)H\(_{16}\)N\(_2\)O\(_5\) 512.0856; found: 513.0935 (M+H\(^{+}\)).

**Cell culture conditions:** The HeLa human cervix adenocarcinoma cells were purchased from the German Resource Centre for Biological Materials (DSMZ) and were grown in DEM supplemented with 10% FBS. Cells were maintained at 37 °C in an atmosphere of 5% CO2 and 95% air and underwent passage twice in a week.

**WST-1 cell viability assays:** Cells were cultured in sterile 96 –well plates at a seeding density of 2.5 x 10\(^3\) cells/well for HeLa and were allowed to settle for 24 h. Dosimeters D1, D2, D3 and D4 were added to the cells at final concentrations of 10, 20, 30 and 50 \( \mu \)M. After 23 h, WST-1 (7 \( \mu \)L of a 50 mg/ml solution) was added to each well. Cells were further incubated for 1 h (a total of 24 h of incubation was therefore studied). Then shaken thoroughly for 1 min. on a shaker and the absorbance was measured at 450 nm against a background control as blank using a microplate ELISA reader. The reference wavelength is 690 nm.

**Live confocal microscopy:** Hela cells were seeded in 24 mm glass coverslips in 6-well plates at a seeding density of 10\(^5\) cells/well. After 24 hours, cells were treated with D1 and D3 at a final concentration of 10 and 30 \( \mu \)M, respectively. After 30 minutes, the medium was removed to eliminate compounds and washed with PBS. Then a solution of Na\(_2\)S in PBS was added at a final concentrations of 0, 100, 200, 500 \( \mu \)M and cells were incubated during 1h at 37 °C. After that, slides were washed twice with PBS to remove traces of the dosimeters. Then slides were visualized under a confocal. Confocal microscopy studies were performed by Confocal Microscopy Service (CIPF). The images were acquired with a Leica TCS SP2 AOBS (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) inverted laser scanning confocal microscope using oil objectives: 63X Plan-Apochromat-Lambda Blue 1.4 N.A. The excitation wavelengths were 450 nm (argon laser) for both dosimeters. Two-dimensional pseudo colour images (255 colour levels) were gathered with a size of 1024 x 1024 pixels and Airy 1 pinhole diameter. All confocal images were acquired using the same settings and the distribution of fluorescence was analyzed using the Image J Software. Identical experiments were done three times to obtain reproducible results.
Acknowledgements

Financial support from the Spanish Government (Project MAT2012-38429-C04-01) and the Generalitat Valenciana (Project PROMETEO/2009/016) is gratefully acknowledged. S.E. is grateful to the Generalitat Valenciana for his Santiago Grisolia fellow. L.E.S.F. also thanks the Carolina Foundation and UPNFM-Honduras for his doctoral grant.

References


